PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
CL000895-PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 00/ 28888	08/11/2000	24/11/1999
Applicant		
PE CORPORATION (NY) et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 7 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [X] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [X] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   NUCLEOTIDE SEQUENCE OF THE SHRIMP WHITE SPOT SYNDROME BACILLIFORM VIRUS (WSBV), SYSTEMS CONTAINING THIS SEQUENCE AND DETECTION KITS

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PC[redacted]S 00/28888

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/01 C12Q1/68 C12N15/12 C12N5/10 A61K38/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE EMBL [Online] 2 November 1999 (1999-11-02) VAN HULTEN, M.C.W. AND VLAK, J.M.: "White spot syndrome virus small subunit of ribonucleotide reductase gene, partial cds, large subunit of ribonucleotide reductase (RR1) gee, complete cds; and unknown genes" XP002166339 Accession AF099142 --- -/--	1,2,4,5, 21-23

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 May 2001	31. 7. 01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	ALCONADA RODRIG.., A

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KIM C K ET AL: "Development of a polymerase chain reaction (PCR) procedure for the detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimp." JOURNAL OF FISH DISEASES, vol. 21, no. 1, January 1998 (1998-01), pages 11-17, XP000997469 ISSN: 0140-7775 page 14, left-hand column, paragraph 1; figure 3 ---	1,2,4,5, 8,9,13, 21-24
A	EP 0 785 255 A (WANG CHUNG HSIUNG ;LU CHU FANG (TW); KOU GUANG HSIUNG (TW)) 23 July 1997 (1997-07-23) figure 15 page 7, line 52 -page 8, line 47 ---	8,9,13
A	NADALA E CESAR B JR ET AL: "A comparative study of three different isolates of white spot virus." DISEASES OF AQUATIC ORGANISMS, vol. 33, no. 3, July 1998 (1998-07), pages 231-234, XP000989432 ISSN: 0177-5103 figure 2 ---	12
A	LO CHU-FANG ET AL: "Specific genomic DNA fragment analysis of different geographical clinical samples of shrimp white spot syndrome virus." DISEASES OF AQUATIC ORGANISMS, vol. 35, no. 3, 26 February 1999 (1999-02-26), pages 175-185, XP000878518 ISSN: 0177-5103 figures 1-4 -----	13,24-39

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PC[redacted] 00/28888

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0785255 A	23-07-1997	US 5824535 A	20-10-1998
		US 6190862 B	20-02-2001

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-39 (partially)

An isolated polypeptide comprising the sequence of SEQ ID NO:3; variants and orthologs thereof; an antibody that binds to said polypeptide; a method for producing and a method for detecting the polypeptide; a method to identify a modulator of the activity of the polypeptide; a method to identify an agent that binds to the polypeptide of the invention; a method of treating WSBV infection sing a compound identified using said method; the nucleic acid molecule of SEQ ID NO:2 encoding said polypeptide; a gene chip comprising said nucleic acid molecule; vector, host cells, transgenic animals comprising said nucleic acid molecule; a method for detecting the polynucleotide sequence; ; a detection reagent comprising said polynucleotide sequence.

2. Claims: 1-39 (partially)

Inventions 2-150 are as invention 1 but referring to the polypeptide sequence of SEQ ID NO:5, 7, 9...281,283 and 285 and to the polynucleotide sequence of SEQ ID NO: 4, 6, 8 ... 280, 282 and 284, 286-293.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 3,17,18 (completely) and 25-39 (partially)

Present claim 3 relates to an antibody defined by reference to a desirable characteristic or property, namely, its ability to bind selectively to the polypeptide of SEQ ID NO:3. The claims cover all antibodies having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for none of such antibodies. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the antibody by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has not been carried out for this claim.

Present claim 17 relates to pharmaceutical compositions comprising an agent defined by reference to a desirable characteristic or property, namely, their abilty to be identified by an assay in which the polypeptide of SEQ ID NO: 3 is contacted with said agent an detecting the formation of a complex between the polypeptide and the agent. The claims cover all agents having this characteristic or property, whereas the application provides neither support within the meaning of Article 6 PCT nor disclosure within the meaning of Article 5 PCT for any of said agents. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the agent by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been not been carried out for this claim which appear to be clear, supported and disclosed. A similar argument is applicable to the method of treatment of a WSBV infection of claim 18 comprising administering a pharmaceutically effective amount of the agent to an organism.

Present claims 25-39 relate to a nucleic acid detection kit defined by reference to a desirable characteristic or property, namely, its ability to detect the presence of one or more genes of WSBV. The claims cover all kits having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT for only a very limited number of such kits. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the detection kit by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the detection kits comprising the polynucleotides of SEQ ID NO:2,4,6...280,284,286-293.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/28888

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 18 is directed to a method of treatment of the animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 3,17,18 (completely) and 25-39 (partially)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-39 partially

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

XP-002166339

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XX
SV   AF099142.1
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DT   21-FEB-2000 (Rel. 62, Last updated, Version 3)
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DE   cds; and unknown genes.
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RL   J. Gen. Virol. 81:307-316(2000).
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RL   Department of Virology, Agricultural University Wageningen, Binnenhaven 11,
RL   Wageningen 6709 PD, The Netherlands
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Development of a polymerase chain reaction (PCR) procedure for the detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimp

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Abstract

The causative viral agent was purified from diseased shrimp *Penaeus japonicus* with white spot syndrome (WSBV). Several hundred clones were obtained from libraries of the purified viral genomic DNA. According to the results of nucleotide sequence analysis, none of the WSBV clones showed considerable sequence homology with those of other known viruses, indicating that WSBV is a new virus causing a serious disease in shrimp. Based on the sequence data of WSBV genomic DNA, a pair of polymerase chain reaction (PCR) primers was designed. After 30 cycles of PCR amplification of viral genomic DNA extracted from WSBV, a single product of the expected size was detected. Southern blot hybridization confirmed that the amplified product was specific to the DNA of WSBV. The PCR system was able to detect 1 pg of WSBV DNA after 30 cycles, and efficiently amplify the target region of WSBV gene in the total nucleic acids extracted either from the diseased shrimp or hatchery shrimp with no signs of viral infection.

Introduction

Recently, disease outbreaks have caused mass mortalities among cultured penaeid shrimps worldwide, especially in Asian countries. The disease in question is characterized by obvious white spots on the carapace, appendages and the inside surface of the body, and also signs of lethargy and red colouration of the hepatopancreas (Inouye, Miwa, Oseko, Nakono & Kimura 1994; Momoyama, Hiraoka, Nakano, Koube, Inouye & Oseka 1994; Nakano, Koube, Umezaea, Momoyama, Hiraoka, Inouye & Oseko 1994; Takahashi, Itami, Kondo, Maeda, Fujii, Tomonaga, Supamattaya & Boonyaratpalin 1994). Most species of economically important shrimps such as giant tiger prawn, *Penaeus monodon*, redtail prawn, *P. penicillatus*, and kuruma shrimp, *P. japonicus*, are affected by the virus.

The causative viral agent, named as baculovirus associated with white spot syndrome (WSBV) was purified from diseased *P. monodon* and histologically characterized (Chou, Huang, Wang, Chiang & Lo 1995; Wang, Lo, Leu, Chou, Yeh, Chou, Tung, Chang, Su & Kou 1995). It is fusiform or rod-shaped with a size of 70–150 nm at its broadest point and a length of 250–380 nm. Therefore, this virus is also known as rod-shaped nuclear virus of *P. japonicus* (RV-PJ) (Inouye *et al.* 1994). The genome of WSBV is a double-stranded DNA molecule longer than 150 kbp (Wang *et al.* 1995). Based on the morphological characteristics and genomic structures of the virus, WSBV was classified into a member of the genus non-occluded baculovirus (NOB) of the sub-family Nudibaculovirinae of the Baculoviridae (Wang *et al.* 1995).

Because of increases in the culture of penaeid shrimp worldwide and because of the impact of WSBV infections on the economic survival of shrimp farms, development of sensitive and rapid

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diagnostic methods are urgently needed to prevent the disease or to control its spread. The simplest method to detect WSBV infection in shrimp is to observe local lesions and white spots on the carapace (Chou *et al.* 1995; Wang *et al.* 1995). Unfortunately, however, no differences between infected and uninfected tissues, or white spots, can be observed in the early stages of infection, especially in hatchery shrimp. Moreover, because virus isolation using shrimp cell lines has not been successful, cell culture techniques cannot be applied at present for the detection of the virus. The only diagnostic tool available for WSBV until recently is to demonstrate virions in shrimp tissues histochemically using an electron microscope (Chou *et al.* 1995). However, this is time consuming, and requires highly trained technical personnel and costly laboratory facilities. Polymerase chain reaction (PCR) is a powerful technique for amplification of DNA (Innis, Gelfand, Sninsky & White 1990), which has been applied for the detection of infectious haematopoietic necrosis virus of salmonid fish (Arakawa, Deering, Higman, Oshima, O'Hara & Winton 1990).

In the present paper, we report studies on WSBV isolated from *P. japonicus* cultured in Korea in an attempt to obtain the genomic sequence of WSBV and understand its correct taxonomic position. This study also aimed to develop a rapid and sensitive diagnostic method, based on PCR amplification, to detect early stages of infection, as well as a latent or carrier state of viral infection.

Materials and methods

Preparation of viral nucleic acids

Viral nucleic acids were prepared as follows. Experimental shrimp in cold TN buffer (20 mM Tris-HCl, 0.4 M NaCl, pH 7.6) was homogenized on ice using a Potter tissue blender. The homogenized solution was centrifuged for 10 min at 1000 *g* to remove cell debris and nuclei. The supernatant from the first centrifugation was layered onto a 20–50% (w/w) sucrose gradient and centrifuged for 30 min at 100 000 *g*. The visible viral band in the middle of the gradient was removed, diluted threefold with TN buffer and pelleted by centrifugation for 30 min at 100 000 *g*. The pellet was resuspended with 500 µl cold TN buffer and incubated with 0.5% SDS, 25 mM EDTA and proteinase K (0.5 mg ml^{-1}) at 55°C for 1 h. The DNA was then subjected to phenol-chloroform extraction and ethanol precipitation. Total genomic DNAs from either infected or uninfected shrimps were prepared directly from homogenized samples by the procedure described above.

Library construction and partial cloning of the genome

For library construction, the WSBV DNA was partially digested with *Sau3*A (Promega), ligated to phagemid (pTZ18R) that had been digested with *Bam* HI, and then used to transform *E. coli* DH5α competent cells prepared as described by Sambrook, Fritsch & Maniatis (1989). Recombinant plasmid DNAs were isolated by alkaline lysis method of Birnboim & Doly (1979).

Southern hybridization

For slot blotting hybridization, plasmid DNAs were spotted onto nitrocellulose membrane filters using slot blot manifolds (BRL) and bound to the membrane by UV crosslinking (Kafatos, Jone & Efstratiadis 1979). For analysis of PCR amplified products, the nucleic acids in the gel were transferred to a nylon membrane (Hybond, Amersham International, Amersham, England) by diffusion blotting as described by Sambrook *et al.* (1989). Probes were prepared by random-priming (Feinberg & Vogelstein 1983) and hybridization was performed as described by Sambrook *et al.* (1989).

DNA sequencing and sequence analysis

Nucleotide sequences were determined by Sanger's dideoxy chain termination method (Sanger, Nicklen & Coulson 1977). For sequence homology comparison, nucleotide sequences of WSBV clones were submitted to the European Bioinformatics Institute (EBI) Worldwide Web site for Fasta homology search (Pearson & Lipman 1988) with the EMBL nucleotide sequence database.

PCR amplification

Two DNA oligonucleotide primers for PCR amplification of the WSBV genome were designed based on sequence data of the WSBV DNA. The sequences of 1F and 1R are 5' ATC TGA TGA GAC AGC CCA AG 3' and 5' GGG AAT GTT AAA TAT GTA TCG G 3', respectively. Polymerase chain reaction amplifications were carried out in a final

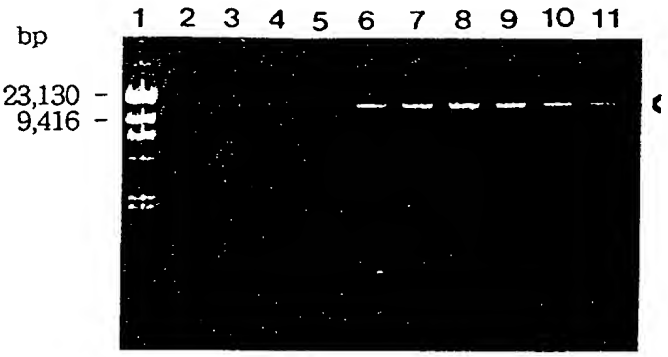

Figure 1 Ethidium bromide-stained agarose gel of WSBV DNA extracted from purified virions. A single molecule of DNA is observed in the gel. Lanes: (1) λ phage DNA Hind III fragment marker; and (2–11) extracted WSBV DNA from each of 11 respective preparations.

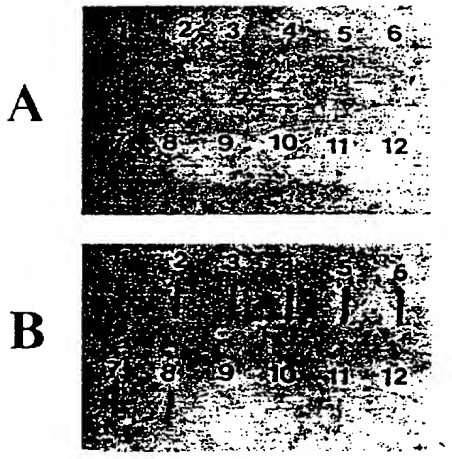

Figure 2 Slot blotting analysis of WSBV recombinant clones probed with either (A) uninfected total DNA or (B) infected total DNA. Key: (1–11) different clones from a WSBV genomic library; and (12) negative control pTZ18R.

volume of 40 µl of a reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM of each dNTP, 15 pmol of each primer, approximately 10 ng of template if not otherwise stated and one unit of *Taq* DNA polymerase. A drop of mineral oil was added to cover the reaction mixture which was amplified for 30 cycles (94°C for 30 s, 55°C for 45 s and 72°C for 45 s) in a DNA thermal cycler (ThermoJeT, Cetus Corp., USA).

Results

Virus purification and genomic DNA isolation

Samples of moribund *P. japonicus* (average weight 20 g) were collected from shrimp farms located in western Korea in 1995. The samples were examined by gross anatomy, and light and electron microscope for confirmation of the disease. Purification of the WSBV virions from infected shrimp was carried out as described in 'Materials and methods'. The visible viral band formed in the middle of the gradient was removed and used for viral DNA preparation. A small volume of purified viral suspension was examined under electron microscopy. The shape and size of the viruses were similar to those observed by Chou *et al.* (1995). Furthermore, the size of genomic DNA isolated from the purified virions was similar to that of WSBV described by Chou *et al.* (1995), estimated to be longer than 150 kbp (Fig. 1), suggesting that the virus described in this study is WSBV.

Cloning of the *Sau3A* digested genomic pieces

Several hundred recombinants were obtained from the phagemid library, constructed as described in 'Materials and methods', and characterized. To confirm that the recombinants contained viral genomic sequences, slot blot hybridization was performed. When probes prepared from total genomic DNA of either infected or uninfected shrimp were used, most of the clones strongly hybridized only with a probe from the infected one (Fig. 2). Therefore, it could be concluded that our recombinants definitely contained some part of the viral genomic DNA which was derived from the virus causing a fatal disease in shrimp. One of the positive clones showing the strongest signal, S1 (Fig. 2B, lane 5), was used for further studies.

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                                    1F
71 GATGAAAAAT ACGATGAATT ATTAGAAGAT AAGGTTGAAG AAATGAGACC GGATATAATC AATGAAGCAT
141 CCGAAACATA TGATAACTTG CTGCTGATAT GATAAGAGAG GTAGACACTA GTAGTGTTAT TGCTCCTGCA
211 ATAGCTGGCA CAGTGGCAAG AACTATCAAT AATTTAAGAG ATAAAAGGAA AGAATATGAA AAGAGGCTAT
281 GGACATTAGC CTACAAACCA TGGAGAAGAT ATGTACAAGC AATTACAGTG ATGGAATTTC GTTTATCATA
351 TAAAGACCTG ACTGTCCATG CCAATTCCGA TACATATTTA ACATTCCCTT TTTTAAGAAT AAAAAAGATC
                                    1R
```

Figure 3 Nucleotide sequence of the WSBV recombinant clone S1. Bold letters indicate a potential initiation site of polypeptide coded by S1, as analysed by Fickett's method. Locations of the primers used for PCR are underlined. The NCBI/GenBank Data Libraries accession number of this sequence is U89843.

DNA sequencing and sequence analysis

One of the recombinant clones which had a 420-bp-insert was fully sequenced and is shown in Fig. 3. According to the results of nucleotide sequence comparison, the sequence from this clone (and also from other clones sequenced so far) showed no significant sequence homology with those of other known viruses or organisms (data not shown), indicating that WSBV is a new virus causing a serious disease in shrimp. Potential coding regions of S1 were searched by Fickett's methods using the PC/Gene program. As a result, 84 amino acids could be coded from nucleotide 196 of S1. Consistently with nucleotide sequence analysis of S1, no polypeptide either from viruses or organisms were identified to have significant amino acid sequence homology with S1.

PCR amplification of purified viral DNA

Initially, 10 ng of the WSBV genomic DNA extracted from purified virion particles was amplified by PCR using a pair of primers (1F and 1R). The amplified product was analysed by agarose gel electrophoresis (Fig. 4). A single product of 365 bp corresponding to that obtained from a positive control DNA S1 (lane 2), was produced from DNA of WSBV purified either from *P. japonicus* (lane 3) or *P. chinensis* (lane 4). This suggests that the same virus infected both species of shrimps, resulting in similar clinical signs.

Sensitivity of PCR tests

The detection limit of WSBV DNA by PCR amplification was examined using 10-fold serially diluted samples of DNA ranging from 10 ng to 10 fg. After 30 cycles of amplification using primers 1F and 1R, a single species of DNA product was detected from samples containing

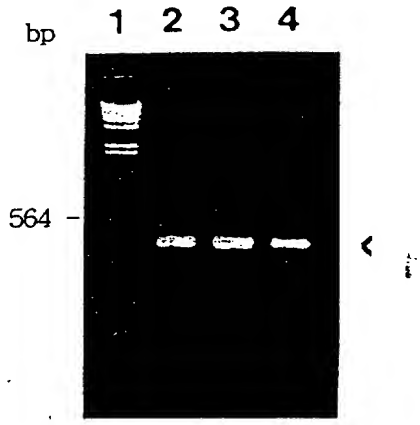

Figure 4 Agarose gel electrophoresis of the PCR product of WSBV DNA from purified virions. Lanes: (1) λ phage DNA Hind III fragment marker; (2) a positive control plasmid pS1; (3) WSBV DNA from *P. japonicus*; and (4) WSBV DNA from *P. chinensis*.

more than 1 pg of WSBV DNA, but not from samples with 100 fg or less (Fig. 5). By increasing the number of PCR incubation cycles to 40, it was possible to detect a smaller quantity of WSBV, down to a level of approximately 10 fg (data not shown).

Direct detection of viral DNA from infected shrimp

Total nucleic acids extracted from either diseased or normal shrimp were subjected to amplification by PCR (Fig. 6). The amplification using nucleic acids from diseased *P. japonicus* (lane 4) or *P. chinensis* (lane 5) yielded a large amount of 365-bp DNA product corresponding to that obtained from WSBV DNA (Fig. 6A). A specific product was obtained from samples containing 1

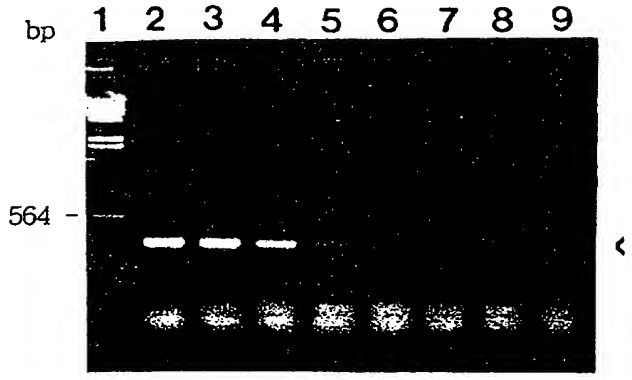

Figure 5 Detection limit of WSBV DNA by PCR amplification with 30 cycles of incubation. Amplified products in agarose gel were stained with ethidium bromide. Lanes: (1) 10 ng of WSBV DNA; (2) 1 ng; (3) 100 pg; (4) 10 pg; (5) 1 pg; (6) 100 fg; and (7) 0 g.

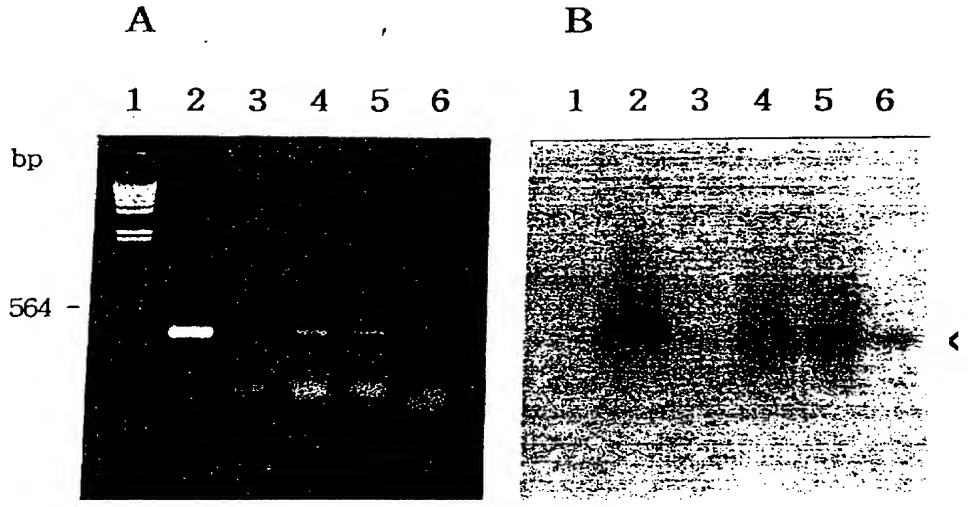

Figure 6 Detection of WSBV DNA from infected shrimp by PCR amplification: (A) agarose gel stained with ethidium bromide: and (B) Southern blot hybridization with [α-^{32}P] dCTP-labelled WSBV DNA from plasmid pS1. Lanes: (1) λ phage DNA Hind III fragment marker; (2) a positive control plasmid pS1; (3) total genomic DNA from uninfected *P. japonicus*; (4) total genomic DNA from infected *P. japonicus*; (5) total genomic DNA from infected *P. chinensis*; and (6) total genomic DNA from hatchery *P. japonicus*.

pg of total nucleic acids from infected shrimp by 40 cycles of reaction. Under the same condition, no product was detected from a nucleic acid sample from a normal shrimp (lane 3). Therefore, the product was evidently derived from DNA of WSBV in the infected shrimp.

To confirm the specificity of PCR products, all of these amplified products were hybridized with the [α-^{32}P] dCTP-labelled probe of S1 from whose sequence PCR primers were derived. As expected, all the PCR products were specifically hybridized with WSBV probe S1 (Fig. 6B), confirming the proper design of primers to detect WSBV DNA.

Diagnosis of hatchery shrimp for viral infection

In addition to being useful for the detection of WSBV from severely diseased shrimp, the target sequence of WSBV DNA was also amplified by

Figure 7 Detection of WSBV DNA from white spots on the carapace of diseased shrimp. Lanes: (1) λ phage DNA Hind III fragment marker; (2) a positive control plasmid pS1; and (3) a white spot from diseased shrimp.

PCR from nucleic acids of potentially infected hatchery shrimp with a length of approximately 1 cm, from which no signs of infection could be detected by other diagnostic methods (Fig. 6, lane 6). Therefore, it is sensitive enough to diagnose WSBV infection in hatchery shrimp.

Detection of WSBV DNA from white spots on the carapace of diseased shrimp

As diseased shrimp with WSBV is well characterized by the spots on the carapace, we investigated what the spots contain and how they are formed. When material from a white spot on the carapace was obtained with a needle and used directly as a template for PCR, the same product was amplified, indicating that it contains WSBV virions (Fig. 7). Therefore, white spots might be derived from the epidermis underneath the carapace, where viral amplification is vigorous, and damaged cells containing virion particles detach and accumulate on the carapace.

Discussion

The causative viral agent purified from diseased *P. japonicus* with white spot syndrome was characterized. According to the results of nucleotide sequence analysis, none of the WSBV clones showed considerable sequence homology (over 60% sequence identity) with those of other known viruses, indicating that WSBV is a novel virus causing a serious disease in shrimp. The nucleotide sequence of WSBV described in this manuscript may not be enough to identify WSBV and efforts are continuing to sequence most of the other clones to obtain further information about the virus.

It is usually impossible to treat affected individuals for viral disease, especially when massive numbers are involved, as in the case of cultured shrimp. Therefore, it is necessary to develop a proper diagnostic method. A PCR amplification method using primers of WSBV DNA, as described in this paper, could specifically detect virus infection in diseased shrimp. It was also possible to detect WSBV DNA from the spots on the carapace of the diseased shrimp. This method was also sensitive enough to specifically amplify the target region of WSBV in hatchery shrimp, even though they did not show any signs of viral infection. The method is also very convenient as it is possible to test total genomic DNA preparations of shrimp, without purification of virions from the infected tissues, and it could be used as a rapid and sensitive diagnostic tool to detect the latent or carrier state as well as an early stage of viral infection.

Recently, several investigators reported similar diagnostic methods for the detection of WSBV from shrimp (Kimura, Yamano, Nakamo, Momoyama, Hiraoka & Inouye 1996; Lo, Leu, Ho, Chen, Peng, Chen, Chou, Yeh, Huang, Chou, Wang & Kou 1996). As the clones described in this paper do not show nucleotide sequence homology with their isolates, these might represent different fragments of WSBV genomic DNA. Also, it cannot be excluded that these derived from slightly different viruses with no significant sequence homology. More sequence analysis of WSBV must be performed to clarify this question.

White spot syndrome DNA was detected from a species of crab, *Sesarma (Parasesarma) pictum*, by a similar procedure to that used to detect virus in shrimp (C K Kim, unpublished results). As this species of crab is one of the most common residents of shrimp farms, it might transmit virus to shrimp by acting as a vector. Therefore, it might be possible to minimize economic loss in shrimp farms by starting shrimp culture with uninfected hatchery shrimp after diagnosis with PCR and removing a potential vector(s) such as this crab during cultivation.

Acknowledgment

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(54) Identification, purification and detection of WSBV (baculovirus associated with white spot syndrome)

(57) This invention relates to the identification, purification and detection of a new infectious viral agent in arthropods, especially shrimps. The virus is named as WSBV (Baculovirus associated with white spot syndrome). Two WSBV genomic DNA libraries were constructed and based upon the sequence of one of the cloned WSBV DNA fragments, a WSBV specific primer set for PCR to detect the WSBV infection in penaeid shrimps has been developed. The results of the present invention provide an effective diagnostic tool for screening WSBV infection in animal host organisms, in particular shrimps, to prevent the further spread of this viral disease.

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Description

FIELD OF INENTION:

This invention relates to the identification, purification and detection of a new infectious viral agent in arthropods, especially shrimps. The virus is named as WSBV (Baculovirus associated with white spot syndrome).

BACKGROUND OF THE INVENTION:

Recently, disease outbreaks have caused mass mortality among cultured penaeid shrimps in Asian countries. Since 1992, outbreaks of a new disease leading to serious mortality among populations of cultured kuruma shrimp (Penaeus japonicus) have occurred in northern Taiwan. The disease is characterized by obvious white spots on the carapace, appendages and the inside surface of the body, and cumulative mortality reaches 100% within 2-7 days. The diseased shrimps also display signs of lethargy and reddish coloration of the hepatopancreas. In 1993, white spot syndrome (W.S.S.) in cultured giant tiger prawn (P. monodon) and redtail prawn (P. penicillatus) was observed. Serious damage to penaeid shrimp production by W.S.S. in Taiwan has been reported (Tung et al. personal communication).

An epizootiological survey of kuruma shrimp in Japan reports similar findings (Nakano et al., Fish Pathology, 29 (2):135-139, 1994). Acccording to the evidence from electron microscopy and the results of challenge tests with the filtrate from diseased shrimp lymphoid organs, the causative agent was a virus that was temporarily designated RV-PJ, a rod-shaped nuclear virus of Penaeus japonicus (Inouye et al., Fish Pathology, 29 (2):149-158, 1994; Takahashi et al., Fish Pathology, 29 (2):121-125, 1994).

To date, the prevalence of baculoviruses in cultured penaeid shrimps has been well documented (Lightner et al., Aquaculture, 32: 209-233, 1983). Among these penaeid baculoviruses, monodon baculovirus (MBV), baculoviral midgut necrosis virus (BMNV) and Baulovirus penaei (BP) were considered to be the most important because they have on occasions caused serious losses in infected shrimp populations (Couch, Nature 247 (5438): 22-231, 1974; J. Invertebr. Pathol., 24:311-331, 1974; Lightner & Redman, J. Invertebr. Pathology, 38: 299-302, 1981; Lightner et al. (1983), supra; Lightner et al., 1987; Sano et al., Fish Pathology, 15: 185-191, 1981).

Baculovirus-like viral particles were observed in the spontaneously diseased penaeid shrimp with W.S.S. (Tung et al., personal communication). This virus may possibly be the main causative agent for the W.S.S. that has occurred in Taiwan in penaeid shrimps. To prevent the spread of W.S.S. in shrimps, thereby rescuing the financial losses caused by this viral disease, it is necessary to identify the actual causative agent and then to develop a diagnostic method that is easy, accurate and not time-comsuing in the detection of W.S.S. without sacrificing the whole subjects tested.

SUMMARY OF THE INVENTION:

This invention is based on the finding of a new causative agent responsible for the incidence of white sopt syndrome in penaeid shrimps. The causative agent has been isolated and purified and found to be a non-occluded rod-shaped virus particle, which is enveloped, 330 ± 20nm in length and 87 ± 7nm in diameter. This virus is determined to be a member of genus NOB (Non-Occluded Baculovirus) of the subfamily Nudibaculovirinae of Baculoviridae and the present isolate is designated as PmNOBIII, and as WSBV (Baculovirus associated with White Spot syndrome) to indicate PmNOBIII related agents. A WSBV genomic DNA library was constructed and based upon the sequence of one of the cloned WSBV DNA fragments, a WSBV specific primer set for PCR to detect the WSBV infection in penaeid shrimps has been developed. By PCR with the WSBV specific primer set, it was demonstrated that the causative agents of white spot syndrome in different shrimp species are in fact closely related. The results of the present invention provide an effective diagnostic tool for screening WSBV infection in animal host organisms, in particular shrimps, which tool may be extremely important in preventing the further spread of this viral disease. An easy, sensitive and specific ready-to-use diagnostic product, which includes primers established based on the nucleotide sequence of a unique genomic DNA clone derived from WSBV, can be developed for the detection of the presence of WSBV and to halt the further spread of this viral disease.

Features and advantages of the present invention will become apparent in the following detailed description of the the preferred embodiments, with reference to the accompanying drawings, of which:

BRIEF DESCRIPTIONS OF THE DRAWINGS:

Fig. 1 is a photograph of Penaeus monodon with white spot syndrome showing the white spots from barely visible to 3 mm in diameter. Bar: 1cm.

Fig. 2 is a light micrograph of cuticular epidermis under the cephalothorax exoskeleton (C) from Penaeus monodon with white spot syndrome showing basophilic inclusions in hypertrophied nuclei of degenerated cells (arrows). Bar:

10 μm.

Fig. 3 is a transmission electron micrograph of thin-sectioned infected tissues underneath the cephalothoracic exoskeletal cuticle (C) from Penaeus monodon with whit spot syndrome showing virus particles in the necrotic area and in a hypertrophied nucleus (Arrow). Bar: 0.5. μm.

Fig. 4 shows rod-shaped viral particles in the epidermis of experim ntally inf cted P. japonicus with the filtrat obtained from diseased P. monodon) Scale bar = 200nm.

Fig. 5 is a negatively stained micrograph of the pellet from filtrate of diseased P. monodon epidermis. Virus particles (arrow) with rod-shape morphology were observed. Scale bar = 500nm.

Fig. 6 displays the white spot (arrow) on the removed carapace of experimentally infected P. japonicus.

Fig. 7 shows the cumulative mortalities (%) of P. japonicus (0.08g average weight) experimentally infected by immersion in filtrates from diseased P. japonicus and P. monodon contrasted with healthy shrimp control group(s).

Fig. 8 is a transmission electron micrograph of negatively stained purified virions showing a tail-like projection (P) extending from one end of the virus. Bar: 0.1 μm.

Fig. 9 is a transmission electron micrograph of Negatively stained non-enveloped nucleocapsid showing the cross-striations on the capsid formed by the ring subunits (arrows). The rings align perpendicularly to the longitudinal axis of the capsid. Bar: 0.1 μm.

Fig. 10 shows an ethidium bromide-stained agarose gel of PmNOBIII DNA extracted from purified virions. A singl molecule of DNA is observed in the gel. Lane 1: lamda phage DNA HindIII fragment marker; lanes 2-4: extracted PmNOBIII DNA from each of three respective preparations.

Fig. 11 shows an ethidium bromide-stained agarose gel of PmNOBIII DNA digested with three restriction endonucleases. At least twenty-two DNA fragments (arrows) can be identified in this gel. Lane 1: lamda phage DNA HindIII fragment marker; lane 2: PmNOBIII DNA HindIII fragments; lane 3: PmNOBIII DNA SalI fragments; lane 4: PmNOBIII DNA XhoI fragments; and lane 5: 1 Kb DNA Ladder.

Fig. 12 shows an ethidium bromide-stained agarose gel of PCR-amplified 18S rDNA fragment from shrimp genomic DNA. Two primers for highly conserved regions of the 18S rRNA sequence of decapods, 143F and 145R, were used for the reaction and primed the amplification of the 848-bp fragment from DNA template prepared from the healthy Penaeus monodon (lane 2). Lane 1, pGEM DNA size marker. The size of DNA markers is indicated in base pairs (bp).

Fig. 13 shows a qualitative assessment using PCR and shrimp DNA specific primer set 143F and 145R, for monitoring shrimp DNA contamination in the WSBV genomic DNA preparations. The PCR products were analyzed on a 1% agarose gel. The shrimp DNA contamination is evidenced by the presence of a 848 bp PCR product. Lane 1, pGEN DNA size marker ; lanes 2-6, WSBV genomic DNA preparations as DNA template; lanes 7-8, shrimp genomic DNA prepared from healthy Penaeus monodon (lane 7) and P. japonicus (lane 8) as DNA template; lane 9: without DNA template. The size of the of DNA markers is given in base pairs (bp).

Fig. 14 shows the SalI digested WSBV DNA fragments. WSBV genomic DNA was digested with SalI restriction endonuclease at 37°c for 3 hr. A 5-μl aliquot was analyzed on a 0.8% agarose gel containing ethidium bromide showing the fragments with a size from 15 kbp to less than 1 kbp (lane 2). From the same batch of digested DNA, a 20-μl aliquot was used for WSBV DNA library construction. Lane 1, lambda phage DNA HindIII fragment marker. The size of DNA markers is indicated in base pairs (bp).

Fig. 15A displays a diagram of the SalI-1461 bp DNA fragment cloned in plasmid pms146 and Fig. 15B shows the locations of the primers, which are used for PCR amplication, in the SalI-1461 bp DNA fragment. The 146F1 and 146R1 prime the amplification of a 1447-bp fragment, while 146F2 and 146R2 prime the amplification of a 941-bp fragment. The positions of two EcoRI sites in SalI 1461 bp DNA fragment are also indicated. Fig. 15C shows the detailed nucleotide sequence of the SalI-1461 bp fragment, in which the locations of the two primer sets 146F1/146R1 and 146F2/146R2 and the two EcoRI sites are also indicated. Fig. 15D shows the nucleotide sequences of six primer sets developed from the SalI-1461 bp fragment.

Fig. 16 shows the PCR amplification of WSBV and shrimp DNA specific fragments using DNA templates prepared from WSBV virions purified by sucrose gradient centrifugation. The WSBV specific primers 146F1 and 146R1 which yield a 1447-bp PCR product were used for reactions in lanes 2, 5, 8, and 11. The shrimp DNA specific primers 143F and 145R which yield a 848-bp PCR product were used for reactions in lanes 3, 6, 9, and 12. In lanes 4, 7, 10, and 13, all the primers 143F, 145R, 146F1 and 146R1 were added together in each of the reactions. The PCR products were analyzed on a 1% agarose gel. Lane 1, pGEM DNA size marker; lanes 2-4, PCR products using DNA template extracted from virions purified from diseased shrimp #1 epidermis showing shrimp DNA and WSBV DNA band; lanes 5-7, PCR product using DNA template extracted from virions purified from diseased shrimp #2 epidermis showing only WSBV DNA band; lanes 8-10, PCR product using DNA template extracted from virions purified from diseased shrimp #2 muscle showing intense shrimp DNA and WSBV DNA band; lanes 11-13, PCR product using DNA template extracted from healthy shrimp showing only shrimp DNA band. The size of DNA markers is indicated in base pairs (bp).

Fig. 17 shows the PCR amplification of WSBV and shrimp DNA specific fragments using plasmid pmsl46 and DNA extracts from Penaeus monodon naturallv infected with WSBV as PCR DNA templates. The WSBV specific primers

146F1 and 146R1 were used for reactions in lanes 2, 5, 8 and 11. The PCR product is a 1447-bp fragment. Internal primers specific to 1447-kbp fragment, 146 F2 and 146 R2, were used for the reactions in lanes 3, 6, 9, and 12; they prime the amplification of a 941-kbp fragment. The shrimp DNA specific primers 143F and 145R were used for the reactions in lanes 4, 7, and 10. They prime the amplification of a 848-bp fragment. The amplification products were analyzed on a 1% agarose gel. Lane 1, pGEM DNA size marker); lanes 2-4, plasmid pms146; lanes 5-7, DNA extracts from naturally infected P. monodon; lanes 8-10, DNA extracts from naturally infected P. japonicus; lanes 11 and 12, template-free control reactions. The size of the DNA markers is given in base pairs (bp).

Fig. 18 shows the PCR amplification of WSBV and shrimp DNA specific fragments using DNA templates prepared from Penaeus monodon experimentally infected with WSBV. The primers 146F1 and 146 R1 which yield a 1447-bp PCR product were used for the reaction. The PCR products were analyzed on a 1% agarose gel. Lane 1, pGEN DNA size marker; lanes 2-4, DNA extracts from 3 experimentally infected P. monodon; lanes 5-7, DNA extracts from healthy P. monodon of control group. The size of DNA markers is indicated in base pairs (bp).

Fig. 19. is a Dot hybridization of DNAs extracted from WSBV infected or healthy Penaeus monodon with DIG-labeled 1447-kbp PCR product. The DNAs from 2 WSBV infected shrimp (1 and 2) and 2 healthy shrimp (3 and 4) were blotted in duplicate (A and B) onto the Hybond-N paper and probed with DIG-labeled 1447-bp PCR product. The probe hybridized with the DNAs from the infected shrimp but not with the DNAs from the healthy shrimp.

Fig. 20 is a Southern hybridization of WSBV DNAs from the diseased P. monodon or P. japonicus with DIG-labeled 1447-bp PCR product. SalI digested WSBV DNAs from P. monodon and P. japonicus were blotted onto the Hybond-N paper and probed with DIG-labeled 1447-bp PCR product. The probe hybridized with a 1461-bp fragment of SalI digested WSBV DNA from either shrimp source with equal visual intensity showing their close relatedness. A: ethidium bromide-stained 0.8 % agarose gel; B: the autoradiograph of the Southern blot of gel (A). Lane 1, pGEN DNA size marker; lane 2, genomic DNA SalI fragments of WSBV purified from P. monodon; lane 3 genomic DNA SalI fragments of WSBV purified from P. japonicus.

Fig. 21 shows the PCR amplication of WSBV DNA specific fragments using primer set 146F1/146F2 and DNA template prepared from arthropods collected from epizootic areas. Lane 1: pGEN marker; lane 2: P. monodon; lane 3: P. japonicus; lane 4: crab; lane 5: copepoda; lame 6: insect (Family: Ephydridae); lane 7: positive control, DNA from known diseased shrimp; and lane 8: negative control, sample without addition of template.

DETAILED DESCRIPTION OF THE INVENTION:

Outbreak of a disease causing serious finacial losses among populations of cultured penaeid shrimps, including Penaeus monodon, P. japonicus and P. penicillatus in Taiwan is characterized by obvious white spot on the carapace, appendages and the inside surface of the body. In order to identify the causative agent of white spot syndrome in penaeid shrimps, electron microscope observations of diseased shrimps were conducted. Heathy juvenile kuruma shrimps (P. japonicus) were exposed by immersion to epidermal filtrate from diseased P. japonicus and P. monodon which exhibited marked white spot signs. Challenge tests used this filtrate on different sized kuruma shrimps.

A non-occluded rod-shaped virus particle was found by electron microscopy in the epidermis of both spontaneously and experimentally infected kuruma shrimps. Virions were enveloped, 330 ± 2Onm in length and 87 ± 7nm in diameter. These experimentally infected shrimps resembled the spontaneously affected ones. Direct inoculation of this virus-containing filtrate into fish cell lines showed no cytopathic effect. Cumulative mortalities reached 100% within 5-7 days and were significantly affected by catching and temperature stress.

The close resemblance in external signs and virus morphology between spontaneously diseased and experimentally infected shrimps indicated that the rod-shaped virus may be the main causative agent of the disease in Taiwan characterized by white spot syndrome. For this reason, this viral disease was proposed the name of "White Spot Syndrome" (W.S.S.). Further studies on the causative agent of W.S.S. (White Spot Syndrome associated Virus) isolated from Penaeus monodon in order to know its taxonomic position.

The causative viral agent was purified from diseased shrimp, Penaeus monodon, with white spot syndrome. Negatively stained preparations show that the virus is pleiomorphic. It is fusiform or rod-shaped. In negatively stained preparations, the virion measures 70 to 150 nm at its broadest point and is 250 to 380 nm long. In some virions, a tail-like projection extends from one end. The capsid is apparently composed of rings of subunits in a stacked series. The rings align perpendicularly to the longitudinal axis of the capsid. The genome of the virus is a double-stranded DNA molecule which produces at least 22 HindIII fragments. The full length of the DNA is estimated to be longer than 150 kbp. Based on the morphological characteristics and genomic structures of the virus, it is confirmed that white spot syndrome associated virus (WSSV) is a member of genus NOB (Non-Occluded Baculovirus) of the subfamily Nudibaculovirinae of Baculoviridae and the present isolate is designaetd as PmNOBIII, and as WSBV (Baculovirus associated with White Spot syndrome) to indicate PmNOBIII related agents.

The WSBV may be closely related to hypodermal and hematopoietic necrosis baculovirus (HHNBV) reported as the pathogen of the explosive epidemic disease of prawn (EEDS) in China in 1993-1994 (Cai et al., J. Fish. China, 19:

112-117, 1995) and systematic ectodermal and mesodermal baculovirus (SEMBV) of the black tiger prawn P naeus monodon in Thailand (Wang et al., Dis. aquat. Org., in press, 1995; Wongteerasupaya et al., Dis. aguat. Org., 21: 69-77, 1995).

The principal clinical sign of this new viral disease is the presence of white spots on the exoskeleton and epidermis of the diseased shrimp with varied sizes from barely visible to 3 mm in diameter. Histopathological study demonstrates that WSBV attacks most frequently the cuticular epidermis, as evidenced by the presence in these tissues of the degenerated cells characterized by hypertrophied nuclei (Momoyama et al., Fish Pathol., 29: 141-148, 1994, Chou et al., Dis. aquat. Org., in press, 1995; C.H. Wang et al., 1995). Thus, the white spot syndrome in penaeid shrimp associated with non-occluded baculovirus can be said to be a well-defined disease and in the prepresent studies we used an isolate of WSBV from P. monodon as the starting material to develop a diagnostic tool for the detection of WSBV in shrimps.

To develop a diagnostic tool for the detection of WSBV and related agent infection in shrimps, the virions were purified from black tiger shrimp Penaeus monodon infected with WSBV. Extraction of viral genomic DNA from purified virions was done by treating the virions with proteinase K and cetyltrimethyl-ammonium bromide (CTAB) followed by phenol-chloroform extraction and ethanol precipitation. A qualitative assessment was performed using polymeras chain reaction (PCR) on the viral DNA and primers specific to shrimp genomic DNA for monitoring shrimp DNA contamination in the viral genomic DNA preparations. A WSBV genomic DNA library was constructed and based upon th sequence of the cloned WSBV DNA fragment, a WSBV specific primer set for PCR to detect the WSBV infection in penaeid shrimps has been designed.

Samples which contained WSBV DNA yielded an evident amplification product showing the expected mobility of a 1447-bp DNA fragment, whereas the nucleic acids extracted from tissue samples from clinically healthy shrimp showed no such DNA fragment, thereby confirming the specificity of the WSBV DNA specific primers designed in the present invention. By PCR with the WSBV specific primer set, it has been demonstrated that the causative agents of white spot syndrome in different shrimp species are in fact closely related. Other host orgainsms, including copepoda, crabs and insects are also tested for the presence of this new causative agent and the currently collected experimental data are positive. The results of this invention provide an effective diagnostic tool for screening shrimp for WSBV infections, which may be extremely important in preventing the further spread of this viral disease.

Materials and Methods:

Shrimp. The healthy kuruma shrimps used for challenge tests were obtained from a hatchery and a shrimp farm in southern Taiwan where no viral disease had been reported. All of the kuruma shrimps were maintained at a temperature of 25-28°C aquaria with aeration and fed an artificial, commercially obtained shrimp food twice daily. Diseased shrimps of P. japonicus were collected from a culture farm in northern Taiwan, while samples of moribund penaeid shrimp, P. monodon (average weight: 30g) were collected from shrimp farms located in southern Taiwan in November 1994. The samples were examined by gross anatomy, light and electron microscopies for the confirmation of the disease using the methodologies as described hereunder.

For light microscopy, both normal kuruma shrimps and individuals displaying marked white spot signs were preserved in Davison's fixative (Bell & Lightner 1988). After 48h in Davison's fixative, specimens were transferred to 50% ethanol, and then processed routinely for histology to 5 μm paraffin wax sections, and stained routinely with hematoxylin and eosin (H & E).

For transmission electron macroscopy, sample of epidermis covering the gill chamber underneath the carapac was removed from naturally and experimentally infected live kuruma shrimps, and immediately prefixed in 2.5% glutaraldehyde in 0.1M cold phosphate buffer solution (PBS, pH 7.4) for 2hr at 4°C. Subsequently, samples were washed several times in cold PBS, and then postfixed in 1% osmium tetroxide for 3 hr at 4°C. The samples were dehydrated and embedded in Spurr's resin. Ultrathin sections were prepared on a Richert-jung Ultracut E Ultrotome, and stained with uranyl acetate and lead citrate. The sections were observed with a HITACHI H-600 transmission electron microscope.

Challenge test. The epidermis from infected P. monodon was removed and homogenized in brackish water at 4°C in the ratio of 1:9. After being centrifuged at 8510 x g (Sigma 2K15 rotor 12141) for 5 min, the supernatant was filtered through a 0.45 μm membrane. The filtrate was centrifuged at 14,549 xg (Sigma 2K15 rotor 12139) for 1.5 hr and tne resulting pellet was resuspended in sterilized brackish water before being applied with negative staining. For negative staining, one drop of suspension was mixed with four drops of the mixture of 0.1% bovine serum albumin and 2% phosphotungstic acid (1:2, pH 7.0). The mixture was placed on a 300 mesh grid for 30-60 sec and excess suspension was removed with filter paper. The preparation was allowed to dry before being examined. Result was observed under a HITACHI H-600 transmission electron microscope.

Cytopathology assay. EPC (epithelioma papulosum cyprini), CHSE-214 (chinook salmon embryo), FHM (fathead minnow) and SSE-5 (sockeye salmon embryo) cells were seeded in 24-well microplates. A filtrate was made from the

epidermis of the diseased shrimps and was diluted from 1/20 to 1/12500 in 5-fold dilutions. Diluted solutions were inoculated into the four fish cell lines and these cells were observed over 2 weeks at an incubation temperature of 20°C.

An infection trial was performed using the filtrate of the epidermis from live or frozen naturally infected P. japonicus and P. monodon. The filtrate was diluted 500-750 times in brackish water in order to be used as a waterborne inoculum. Two replicates of thirty-five one-month-old juvenile kuruma shrimps (mean weight 0.08g) were immersed in these diluted filtrates for 2h. Two other populations were similarly exposed, either to the filtrate from healthy P. monodon epidermis or to Grace's insect medium. These served as controls. After inoculation, shrimps were kept in glass aquaria with aeration. Water temperature and salinity were 25-28°C and 25-30 ppt, respectively, throughout the experiment. The mortality was observed daily and the moribund shrimps were collected and examined by transmission electron microscopy.

Purification, Genomic Structure and Taxonomic Position of WSBV

The purification of the virions of WSSV from P. monodon shrimp was carried out as follows. The shrimps were first rinsed with cold 1 x TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.6). The exoskeleton with underlying epidermis taken from 1 to 5 live or frozen shrimp was extracted with 20 ml cold extraction buffer (20mM HEPES, 0.4 N NaCl, 1 mM EDTA, lmM EGTA, 1 mM DTT, 2.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1.6 μg/ml pepstatin, 2 μg/ml aprotinin, 1 μg/ml bestatin), and then purified by centrifugation on linear 35 to 65% (W/W) sucrose gradient at 74,700 xg (Hitachi SRP 28SA rotor at 24,000rpm) for 60 min. The visible viral band in the midway of the gradient was removed and pelleted by centrifugation at 74,700 xg at 4°C for 30 min. The pellet was washed twice with cold 1 x TE buffer, resuspended with 300-500 ml cold 1 x TE buffer depending on the size of the pellet, and immediately used for viral DNA extraction. A small volume of purified virus suspensions was negatively stained with 2% phosphotungstic acid (PTA) at pH 7 for the ultrastructural studies of the virions.

The extraction of viral genomic DNA from gradient purified virions was performed by proteinase K and N-cetyl N, N,N-trimethylammonium bromide (CTAB) treatments followed by phenol-chloroform extraction and ethanol precipitation (K. Wilson (1994), Preparation of genomic DNA from bacteria. Miniprep of bacterial genomic DNA. in Ausubel, F. M. et al. (eds.) Current Protocols in Molecular Biology, Vol.1. Greene Pub. Assoc. and Wiley-Interscience, NY, p. 2.4.1-2.4.5).

The estimation of the viral genome size was done by restriction endonuclease analysis. Viral DNAs were digested with HindIII, SalI and XhoI restriction endonucleases (Boehringer Mannheim Company). Restriction fragments were separated by electrophoresis in 0.8% agarose gel (9 cm x 12 cm), with Tris-acetate buffer (0.04M Tris-acetate, 0.1 mM EDTA, pH 8.0) containing 0.5 μg/ml ethidium bromide. The 1-kilobase (kb) DNA ladder and lambda phage HindIII fragment marker (Life Technologies, Inc.) were used as the DNA size standard on the gel.

Development of Effective Diagnostic Tools.

For the development of effective diagnostic tools, the construction of WSBV genomic library was conducted by cloning "super pure" WSBV genomic DNA extracted from purified virions. In addition, the amplification of selected DNA sequence by polymerase chain reaction (PCR) promises to be a powerful diagnostic tool for the identification of pathogens (Erlich et al., Nature 331: 461-462, 1988; Oste, C., Biotechniques 6: 162-167, 1988). Based upon the sequences of the cloned WSBV DNA fragments, a WSBV specific primer set for PCR has been designed.

I. WSBV genomic DNA library construction

A. Virus purification and extraction of viral DNA

The same batch of the frozen WSBV infected black tiger shrimp Penaeus monodon, as used for the taxominic studies, was the source of the virus, and this strain of the WSBV is named as PmNOBIII (the third non-occluded baculovirus reported for P. monodon) according to the criteria set forth in Francki et al. (Arch. Virol., 2: 1-450, 1991). . The purification of the virions was carried out as described in the previous paragraphs. The extraction of viral genomic DNA from purified virions was performed by treating the virions with proteinase K and N-cetyl N,N,N-trimethylammonium bromide (CTAB) followed by phenol-chloroform extraction and ethanol precipitation (Wilson (1994), supra) . Briefly, the gradient-purified virions were incubated in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6) containing 100 mM KCl, 1% SLS (N-lauryl sarcosine) and 0.2 mg/ml proteinase K at 65°C for 3 hr. After incubation, 5 M NaCl was added to adjust the NaCl concentration of the DNA solution to 0.7 M. Next, 1/10 vol. CTAB/NaCl (10% CTAB in 0.7 M NaCl) was added slowly and mixed thoroughly before incubation at 65°C for 10 min.

Following two extractions with an approximately equal volume of chloroform/isoamyl alcohol and two extractions with an equal volume of phenol/chloroform/isoamyl alcohol, the DNA was precipitated with two volumes. absolute

ethanol, and washed with cold 70% ethanol. The dried DNA pellet was dissolved in a suitable amount of 0.1 x TE buffer at 65°C for 30 min, and then stored at 4°C until use.

B. Preparation of shrimp DNA for PCR as a control

The primers specific to shrimp genomic DNA for PCR were used to monitor shrimp DNA contamination in the WSBV genomic DNA preparations. For this purpose, two primers were designed from the highly conserved regions of 18S rRNA sequence of decapods, based on published sequences (Kim & Abele, J. Crust. Biol., 10, 1-13, 1990), a computerized data file (GenBank, National Institute of Health, MD, U.S.A.) and the sequence alignment analysis using PC/GENE program (Intelligenetics, Inc.). By pairing the forward primer 143F (5'- TGC CTT ATC AGC TNT CGA TTG TAG-3', where N represents G, A, T or C) with a reverse primer 145R (5'-TTC AGN TTT GCA ACC ATA CTT CCC-3'), the shrimp DNA is expected to yield a PCR product of 848 bp corresponding to nucleotide sequences 352 to 1200 of 18S rRNA of P. aztecus.

The genomic DNAs extracted from the muscle of healthy P. monodon or P. japonicus were used as positive control for PCR. The deproteinized genomic DNA of the shrimp was prepared according to the method for preparation of genomic DNA from mammalian tissue (Strauss, WM (1994) Preparation of genomic DNA from mammalian tissue. In Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (Eds) Current Protocols in Molecular Biology, Vol.1. Greene Publishing Associates, Inc. and John Wiley and Sons, Inc., New York, p. 2.2.1-2.2.3). Briefly, 200 mg muscle tissue excised from the abdomen of the shrimp was rapidly frozen in liquid nitrogen and crushed to a fine powder. The processed tissue was placed in 2.4 ml digestion buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8, 25 mM EDTA, pH 8, 0.5% sodium dodecyl sulfate, 0.1 mg/ml proteinase K) and incubated at 65°C for 12 to 18 hr. The digest was deproteinized by successive phenol/chloroform/isoamyl alcohol extractions, recovered by ethanol precipitation, and dried and resuspended in 0.1 x TE buffer at 65°C for 30 min, and then stored at 4°C until use for PCR.

C. WSBV genomic DNA library construction

Two WSBV genomic libraries, PmNOBIII SalI (pms) and PmNOBIII HindIII (pmh) were contructed as set forth below. The WSBV genomic DNA without shrimp DNA contamination was digested with SalI or HindIII restriction endonuclease (BRL, Life Technologies Inc.) at 37°C for 3 hr in order to obtain DNA fragments, and the fragments were then ligated into SalI or HindIII cleaved pUC 19 plasmid vector in the presence of T4 DNA ligase at 16°C overnight. The competent Escherichia coli DH 5α cells were transformed with the resulting plasmids and plated on ampicillin/isopropyl-β-D-thiogalactopyranoside (IPTG)/5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) agar plates. After using miniprep method to screen the white ampicillin-resistant transformants for the presence of the appropriate recombinant plasmids, both strands of the plasmid inserts were sequenced with double-stranded DNA templates using a Sequenase kit (United States Biochemical Corp.) with M13/pUC Sequencing Primers (GIBCO BRL Life Technologies Inc.), and subsequently, specific internal primers. Recombinant plasmids were isolated from transformants and screened for the presence of the insert by SalI or HindIII digestion. The size of the inserts were listed in Table 1.

II. Amplification of WSBV DNA fragment from DNA extracted from purified WSBV virions

Oligonucleotide primers (146F and 146R) are used for the amplification of WSBV DNA fragments. Primers 146F and 146R are designed on the basis of the DNA sequence of a cloned WSBV 1461-bp SalI DNA fragment in recombinant plasmid pms146 and there have been established 6 primer sets as shown in Fig. 15D. The primer set of 146R1 and 146F1 have the following nucleotide sequences: 146R1, 5'-TAA TGC GGG TGT AAT GTT CTT ACG A-3'; 146F1, 5'-ACT ACT AAC TTC AGC CTA TCT AG-3'. With this primer set, a 1447-bp fragment is expected to be amplified from WSBV genomic DNA. The internal primer set, 146R2, 5'-TAC GGC AGC TGC TGC ACC TTG T-3', and 146F2, 5'-GTA ACT GCC CCT TCC ATC TCC A-3' are used to confirm that the amplified fragment is indeed from the WSBV 941-bp SalI DNA fragment.

The deproteinized DNA samples extracted from purified WSBV virions and from the muscle of the healthy shrimp were used as DNA templates for the evaluation of the specificity of the primers by PCR.

III. Amplification of WSBV DNA fragment from DNA extracted from tissues of shrimp naturally and experimentally infected with WSBV

The diseased shrimps consisted of shrimp naturally and experimentally infected with WSBV. For experim ntal infection, the healthy shrimp (average body weight: 0.5 gm) were infected with WSBV using the method described in the preceding paragraphs. Five days after infection, the DNAs were extracted from three experimentally infected shrimp and three healthy shrimp and checked by PCR with the use of WSBV specific primers (146F1 and 146R1) and shrimp

DNA specific primers (143F and 145R).

IV. PCR amplification and analysis of products

The deproteinized DNA samples used for amplification totaled 0.1-0.3 μg in a 100-μl reaction mixture containing 10 mM Tris-HCl, pH 9 at 25°C, 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 200 μM each of dNTP, 100 pmol each of primer, 2.5 units of Taq DNA Polymerase (Promega). The amplification was performed in a AG-9600 Thermal Station (Biotronics Corp.) for one cycle of 94°C for 4 min, 55°C for 1 min, 72°C for 3 min; 39 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 3 min, plus a final 5 min extension at 72°C after 40 cycles. Control reactions containing no template DNAs were run for all PCR reactions. In some PCR reactions, controls also consisted of reaction mixtures with DNA extracts from healthy shrimp. The PCR products were analyzed in 1% agarose gels containing ethidium bromide at a concentration of 0.5 μg/ml, and visualized under an ultraviolet transillumination.

V. Dot hybridization of DNAs extracted from WSBV infected or healthy P. monodon with DIG-labeled 1447-bp PCR product

The DNAs extracted from WSBV infected or healthy P. monodon were spotted onto Hybond-N paper (Amersham) using a 96-well dot-blot vacuum filtration manifold apparatus (Schleicher and Schuell, Inc.). The blots were air dried and denatured in 1.5 M NaCl, 0.5 N NaOH for 10 min, and then neutralized in 1.5 M NaCl, 1 M Tris, pH 7.4 for 10 min. The blots were used for hybridization with a DIG-labeled 1447-bp PCR product following the standard molecular cloning techniques (Sambrook J, Fritsch EF, Maniatis T (1989,) Molecular Cloning: A Laboratory Manual, 2nd. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). The dot blot was hybridized at 37°C for 16 hr with the DIG-labeled probe, after prehybridization at 37°C for 12 hr in 50% formamide, 5X SSC, 1 mM EDTA, 50 mM Tris (pH 8), 5X Denhardt's reagent (0.1% Ficoll-400, 0.1% polyvinyl pyrrolidone, 0.1% BSA). The 1447-bp PCR product was used as a template to prepare probe using the random primer method (Boehringer Mannheim). After hybridization, the detection of the DIG-labeled nucleotides in blots was accomplished with a chemiluminescent reaction by using the DIG Luminescent Detection Kit (Boehringer Mannheim). The blot was exposed to Kodak XAR-5 film at 37°C for 15-30 min to record the chemiluminescent signal.

VI. Southern hybridization of WSBV DNAs from the diseased P. monodon or P. japonicus with DIG-labeled 1447-bp

PCR product

Southern blot hybridization was performed to localized the 1447-bp PCR product within the genomic DNA of WSBV purified from the diseased P. monodon or P. japonicus with white spot syndrome. For this purpose, 200 ng genomic DNA of WSBV isolated from the diseased shrimp was digested with SalI, and then electrophoretically separated in 0.8% agarose gel. After acid (0.25 N HCl) depurination and alkali (1.5 M NaCl-0.5 N NaOH) denaturation of the DNA, the gel was neutralized with 1 M Tris (pH 7.4) and 1.5 M NaCl, and subsequently transferred to a Hybond-N nylon membrane using a vacuum transfer unit (Hoefer TE 80) for 60 min. The 20X SSC (3 M NaCl, 1.5 M Sodium Citrate) was used as transfer buffer (Sambrook et al. (1989), supra). The blot was used for hybridization with a DIG-labeled 1447-bp PCR product.

VII. Detection of WSBV in Arthropods Collected from Epizootic Areas

The DNA templates prepared from arthropods collected from epizootic areas were used for PCR with 146R1 and 146F1 primers for the detection of WSBV in the tested organisms.

RESULTS:

A. Histopathological studies:

Outbreaks of W.S.S. amongst penaeid shrimps is evidently not a confined, local problem anymore. It has already brought the cultured shrimp industry in Asia to a critical condition. In order to classify the causative virus more clearly and develop a quick diagnostic method, further studies on the physicochemical characterization of this agent is conducted.

The main clinical signs of the disease in Penaeus monodon were the white spots on the exoskeleton (Fig. 1). The white spots were particularly obvious on the carapace removed from the diseased shrimp, and were readily observed

even on the carapaces from lightly infected animals. Histopathological study demonstrates that epidermis of the diseased shrimp was attacked by viral agent evidenced by the presence in this tissue of the degenerated cells characterized by hypertrophied nuclei with inclusions (Fig. 2).

Ultrathin sections of the underlying epidermis of the cuticle from shrimp with white spot syndrome viewed under the electron microscope revealed numerous non-occluded baculo-like virus particles in the necrotic areas. The hypertrophied nuclei filled with virions were also readily seen (Fig. 3). The virus particles were 330 ± 20nm in length and 87 ± 7nm in diameter (n=30). The electron-dense central core of the virus particle is nucleocapsid, approximately 220 X 70 nm in size. No difference in virion morphology between spontaneouly diseased and experimentally infected shrimp was recognized (Fig. 4).

B. Negative staining and cytotoxicity assay of the filtrate for challenge test:

The result of negative staining of the pellet from the filtrate of diseased P. monodon epidermis is shown in Fig. 5. Virus particles with rod-shape morphology can be seen. These are similar to the virus particles observed in ultrathin sections of spontaneously diseased shrimps. No bacteria were observed.

Cytopathic effect (CPE) was not found in any of the four tested fish cell lines; the filtrate which was used as waterborne inoculum had no cytotoxicity.

C. Challenge test:

Healthy shrimps were exposed to epidermal filtrate from disesed P. japonicus and P. monodon which exhibited marked white spot symptoms. These experimentally infected shrimps resembled the spontaneously affected ones (Fig. 6) and cumulative mortalities reached 100% within 5-7 days (Fig. 7), while no shrimp died in the control groups.

Further, the inoculum was highly pathogenic to the smallest shrimps tested (mean weight of 0.08g) and all these shrimps died within 5 days; only 35% cumulative mortality was found in the 0.16g-sized shrimp group after 7 days although mortality reached 100% in 12 days; and 10% mortality was observed in the group of largest shrimp (mean weight of 0.26g) within 2 weeks. No shrimp died in the control groups.

D. Purification, Genomic Structure and Taxonomic Position of WSBV:

The purified virions were fusiform or rod-shaped with bluntly rounded ends. In negatively stained preparations, the virion was 70 to 150 nm at its broadest point, and was 250 to 380 nm long, which is usually 10% larger than in ultrathin sections. In some virions, a tail-like projection extending from one end was observed (Fig. 8). The non-enveloped nucleocapsids were normally 58 to 67 nm in diameter and 330 to 350 nm long. The capsid components formed parallel cross-striations (Fig. 9). Thus, the capsid seemed to be composed of rings of subunits in a stacked series. The thickness of the rings (20 nm) was very constant and the rings were perpendicular to the longitudinal axis of the capsid. In term of virus morphology, WSSV resembles SEMBV (Systemic Ectodermal and Mesodermal Baculovirus) and differs from BMN (Baculoviral Mid-gut Gland Necrosis Virus) and PmSNPV (Penaeus monodon Single Nucleocapsid Nuclear Polyhedrosis virus=MBV) (Mari et al., Dis. aquat. Org. 16: 207-215, 1993; Sano et al., Helgolander Meeresunters. 37: 255-264, 1984; Wongteerasupaya et al., Dis. aquat. Org. 21: 69-77, 1995). However, the main clinical sign of white spot caused by WSSV was not described in the SEMBV infected shrimp. To date, it is difficult to guess the relatedness between WSSV and SEMBV.

A single DNA molecule was extracted from purified virions of WSBV (Fig. 10). The genomic DNA of WSBV digested with HindIII, SalI and XhoI restriction endonucleases was shown in Fig. 11. The genomic DNA of WSSV digested with HindIII restriction endonuclease produced, in the agarose gel, at least 22 fragments of approximate sizes: 19.4, 16.9, 14.9, 12.5, 10.0, 9.6, 8.4, 8.0, 7.3, 6.1, 5.5, 4.8 , 4.3, 3.9, 3.6, 3.3, 3.0, 2.5, 2.0, 1.6, 1.4, and 1.1 kbp, respectively. The fragments smaller than 1 kbp have run over the gel if they exist. The length of WSBV DNA was estimated to be longer than 150 kbp, which falls within the size range of 90-230 kbp found in insect baculoviruses (Francki et al., Arch. Virol., suppl. 2: 1-450, 1991).

Based on the morphological characteristics and genomic structure, WSSV is classified as the genus Non-Occluded Baculovirus (NOB) of the subfamily Nudibaculovirinae of Baculoviridae (Francki et al. (1991), supra) and the isolate was named PmNOBIII, as the third non-occluded baculovirus reported for P. monodon (D.V. Lightner, Boca Raton, p. 393-486, 1993; Wongteerasupaya et al (1995), supra). The present virus isolate PmNOBIII was deposited in the China Center for Type Culture Collection (CCTCC) of the People's Republic of China on January 11, 1996 with the Accession number CCTCC-V96001 under the Budapest Treaty. It is also proposed to use WSBV (Baculovirus associated with White-spot Syndrome) to indicate the PmNOBIII related agents.

E. Development of Effective Diagnostic Tools for WSBV Infection:

I. WSBV genomic DNA library construction:

a) Virus purification and extraction of viral DNA:

Typical rod-shaped virions of WSBV were readily observed after concentration and purification by sucrose gradient centrifugation. These virions were used to extract the viral DNA.

The amplification of shrimp DNA using PCR and primers specific to 18S rRNA reliably resulted in a predicted 848-bp DNA fragment (Fig. 12). This provided a simple and highly sensitive method for detecting small amounts of shrimp DNA and was subsequently used to monitor shrimp DNA contamination in WSBV genomic DNA preparations for library construction. The PCR analysis shown in Fig. 13 indicates that host DNA contamination was detected in most WSBV genomic DNA preparations. However, a few samples of WSBV genomic DNA extracted from purified virion preparations were virtually free of contaminating host DNA. An example is shown in Fig. 13, lane 3.

b) Genomic DNA library construction:

Two WSBV genomic libraries, PmNOBIII SalI (pms) and PmNOBIII HindIII (pmh) were contructed with use of SalI or HindIII restriction endonuclease (BRL, Life Technologies Inc.) and pUC 19 plasmid vector.

For example, the SalI digested WSBV DNA was checked by electrophoresing a 5-µl aliquot in a 0.8% agarose gel containing ethidium bromide. The WSBV genomic DNA was completely digested with SalI restriction endonuclease (Fig. 14). From the same batch of digested DNA, a 20-µl aliquot was used for library construction.

Recombinant plasmids isolated from transformants were screened by SalI or HindIII digestion, among which 592 clones (pms1-pms592) from pms library and 410 clones (pmhl-pmh245 and pmh419-pmh584) from pmh library were screened for the presentce of the insert by SalI or HindIII digestion. The size of the inserts varied from 15kbp to less than 100bp as shown in Table 1. These libraries provide an abundant supply of WSBV DNA, enabling further study of the molecular biology of the virus and developemnt of nucleic acid and immunological diagnostic kits.

II. Amplification of WSBV DNA fragment from deproteinized DNA extracted from purified virions:

On the basis of the obtained DNA sequences (data not shown) of WSBV SalI DNA fragments, several primer sets were designed and evaluated by PCR for their ability to identify the WSBV in infected tissues.

Fig. 15A displays a diagram of the SalI-1461 bp DNA fragment cloned in plasmid pms146 and Fig. 15B shows the locations of the primers, which are used for PCR amplication, in the SalI-1461 bp DNA fragment. The 146F1 and 146R1 prime the amplification of a 1447-bp fragment, while 146F2 and 146R2 prime the amplification of a 941-bp fragment. The positions of two EcoRI sites in SalI 1461 bp DNA fragment are also indicated. Fig. 15C shows the detailed nucleotide sequence of the SalI-1461 bp fragment, in which the locations of the two primer sets 146F1/146R1 and 146F2/146R2 and the two EcoRI sites are also indicated. Fig. 15D shows the nucleotide sequences of six primer sets developed from the SalI-1461 bp fragment. Among them, the primer set 146F1/146R1 gave a consistent and an efficient amplification of WSBV DNA but not of shrimp DNA. This primer set was then chosen for subsequent parts of this study.

Figure 16 shows the results of amplification using purified WSBV genomic DNA as PCR template, and the primer sets either specific to WSBV DNA or to shrimp DNA. The reactions analyzed in Fig. 16, lanes 2, 5 and 8 represent amplification using WSBV DNA primer set 146F1-146R1 and three independent WSBV DNA preparations, and the results demonstrate the presence of a relatively large amount of WSBV genomic DNA in the three tested samples, as evidenced by an intense 1447-bp PCR product in these lanes. At least one of the WSBV DNA preparations is free from shrimp DNA contamination, as evidenced by the absence of detectable PCR product of shrimp DNA in Fig. 16, lane 6. The WSBV primer set 146F1/146R1 and shrimp DNA primer set 143F/145R were used simultaneously in a reaction mixture for demonstrating approximately the proportion of WSBV DNA in template DNAs.

The data presented in Fig. 16 demonstrate that WSBV specific DNA fragment was detected as a major band in three independent WSBV preparations (lanes 4, 7 and 10) while the shrimp DNA was detected in two of three WSBV DNA preparations (lanes 4 and 10). Thus template DNAs contained varying proportions of shrimp DNA and WSBV DNA. It is also clear that in spite of contamination with shrimp DNA, a large proportion of the DNAs extracted from WSBV virions purified by sucrose gradient centrifugation is WSBV DNA. Meanwhile, reaction mixtures with total nucleic acid extracted from tissues from clinically healthy shrimp and WSBV DNA specific primer set 146F1/146R1 were consistently negative (Fig. 16, lane 11), thus demonstrating the specificity of this primer set.

III. Amplification of WSBV DNA fragment from DNA extracted from the shrimp tissues naturally and experimentally infected with WSBV:

Figure 17 shows the amplification results using plasmid pmsl46 DNA and the DNA extracted from the tissues of P. monodon and P. japonicus natually infected with WSBV as DNA templates. Th DNA templates were amplified using either the WSBV-specific primer set 146F1/146R1 or shrimp DNA-specific primer set 143F/145R. The 1447-bp PCR product, comigrating with DNA amplified from pure plasmid pms146 DNA, demonstrates the presence of WSBV DNA in the total nucleic acid extracted from all the naturally infected shrimp. Examples are shown in Fig. 17, lanes 2, 5 and 8.

Using the internal primer set 146F2/146R2, 10 µl of these products were reamplified to yield a PCR product with the expected size of 941 bp (Fig. 17, lanes 3, 6 and 9). The results confirm the identity between amplification product and template. Shrimp DNA was amplified very efficiently using shrimp DNA specific primer set 143F/145R as shown in Fig. 17, lanes 7 and 10. The results presented in Fig. 17, lanes 5 to 10 demonstrate that WSBV DNA could be detected with the use of WSBV DNA specific primer sets 146F1/146R1 and 146F2/146R2 in the presence of a large excess of shrimp genomic DNA.

Figure 18 shows the amplication result using DNA extracted from tissues of P. monodon experimentally infected with WSBV as DNA templates for PCR using primer set 146F1/146R1. Amplication of the expected 1447-bp fragment is evident for all the experimentally infected shrimp. No amplification product at 1447 bp was present for healthy shrimp from control group.

IV. Dot hybridization of DNAs extracted from WSBV infected or healthy P. monodon with DIG-labeled 1447-bp PCR product:

The results of dot hybridization demonstrate that the PCR product hybridized with DNAs extracted from WSBV infected shrimp, but did not hybridize with DNAs extracted from healthy shrimp. (Fig. 19). The results demonstrate the specificity of the 1447-bp PCR product.

V. Southern hybridization of WSBV DNAs from the diseased P. monodon or P. japonicus with DIG-labeled 1447-bp PCR product:

In order to localize the 1447-bp PCR product within the WSBV genomic DNA, Southern hybridization of WSBV genomic DNA Sall fragments was performed using DIG-labeled 1447-bp PCR product as a probe. The results demonstrate that 1447-bp PCR product hybridized specifically with a WSBV genomic DNA Sall fragment of 1461 bp (Fig. 20). Both 1461 bp Sall fragments of WSBV genomic DNAs prepared respectively from P. monodon and P. japonicus were found to be positive with the probe.

VI. Detection of WSBV in Arthropods Collected from Epizootic Areas:

Among tested organisms, P. monodon, P. japonicus, crabs, copepoda and insect (Family: Ephydridae) gave WSBV positive results (Fig. 21).

DISCUSSION:

The diseased shrimps have obvious white spots on the carapace, appendages and the inside surface of the body, and also display signs of lethargy and reddish coloration of the hepatopancreas. Vibriosis, virus infection, poor environmental management and nutrient imbalance have all been conjectured to be the possible cause for these outbreaks. Based on electron microscope observation, however, a rod-shaped virus was considered to be the main causative agent. In the present study, the pathogenicity of a pathogenic virus from diseased P. japonicus and P. monodon with white spot syndrome was investigated. Close resemblance in white spot signs and virus morphology between spontaneously diseased and experimentally infected shrimps demonstrated that this virus is indeed the causative agent of the outbreak. The virus is highly pathogenic and constitutes a threat to shrimp. Information pilot studies in which diseased shrimps were fed to healthy specimens suggest that the virus may be transmitted orally as well as via water.

In addition to WSBV, a variety of baculoviruses has been reported to infect decapod crustaceans since the first report by Couch (Nature, 247 (5438):229-231, 1974; J. Invertebr. Pathol., 24: 311-331, 1974) and some of them cause mass mortality of the diseased animals (Lightner & Redman, J. Invertebr. Pathol., 38: 299-302, 1981; Sano et al., Fish Pathol., 15: 185-191, 1981; Lester et al., Dis. aquat. Org., 3: 217-219, 1987; Johnson P.T., Dis. aquat. Org., 5: 111-122, 1988; Johnson & Lightner, Dis. aquat. Org., 5: 123-141, 1988; Bruce et al., J. Virol. mehods, 34: 245-254, 1991; Chang et al., Fish Pathol., 27 (3): 127-130, 1992; Chang et al., J. Invertebr. Pathol., 62: 116-120, 1993; Mari et al., Dis. aquat.

Org., 16: 207-215, 1993, Wongteerasupara et at., Dis. aquat. Org., 21: 69-77, 1995). These viruses are morphologically similar, and most researchers agree that the structure of the viral genome should become the much needed reference for determining the taxonomic position of crustacean baculoviruses. The development of rapid and reliable diagnostic tools using molecular approaches will be useful not only for the identification and comparative studies of the viruses but also for the screening of carriers in shrimp larvae and parental spawners. In view of these points the present researches are focused on the WSBV genomic structure and on the development of rapid and sensitive diagnostic tools.

In experiment, shrimp DNA specific primers are used in several assessments. The aims of the use of shrimp DNA specific primer set in the present study were (i) to assess the purity of WSBV genomic DNA preparations, (ii) to evaluate nucleic acid extraction procedures for yielding amplicable DNA template, and (iii) to estimate approximately the proportion of the shrimp DNA and WSBV DNA in template DNAs prepared from total nucleic acids of the infected tissues. Attempts have been made in our laboratory to purify WSBV virions from various tissues including epidermis, muscle and gills. From these virions we obtained WSBV DNA of varied purity as assessed by shrimp DNA specific primers. Examples of these assessments are shown in Figs. 13 and 17. The nucleic acids extracted from muscle tissues yielded a great quantity of WSBV DNA, but were heavily contaminated with shrimp DNA (Fig. 16, lane 9). The virions purified from heavily infected epidermal cells underneath the exoskeleton are good starting materials for extracting "super pure" WSBV genomic DNA (Fig. 16 lanes 2 and 5). By using the shrimp DNA specific primers and PCR, for the first time a tool is available to assess the extent of the shrimp DNA contamination in shrimp virus genomic DNA preparations.

Using the WSBV DNA specific primers, all the purified WSBV genomic DNA samples consistently yielded an evident amplification product showing the expected mobility of a 1447-kbp DNA fragment. The nucleic acids extracted from tissues of naturally diseased shrimp with white spot syndrome and from shrimp experimentally infected with WSBV also consistently gave PCR products of the same size. The nucleic acids extracted from the tissues of clinically healthy shrimp showed no positive results. These results demonstrate the specificity of the WSBV DNA specific primers designed in the present study. In addition, the 1447-bp PCR product can be used to prepared WSBV specific nucleic acid probe for detecting WSBV infection in shrimp using dot blot hybridization as shown in Fig. 19. Practically, the present studies provide three effective diagnostic tools for screening of the WSBV infection in penaeid shrimps as shown in Figs. 17, 19 and 20.

With PCR (Fig. 17) and Southern hybridization (Fig. 20), we have demonstrated that the causative agents of white spot syndrome of different shrimp species are in fact closely related. Screening for the WSBV infection in shrimp should be undertaken immediately in order to prevent this viral disease from spreading further. On the other hand, the PCR diagnostic techniques for WSBV developed in the present study provide effective tools for the comparative studies on the shrimp non-occluded baculoviruses such as Japan' RV-PJ (Inouye et al (1994), supra), China' HHNBV (Cai et al., J. Fish. China, 19: 112-117, 1995), Thailand' SEMBV (Wongteerasupaya et al. (1995), supra), the present WSBV isolate PmNOBIII and other crustacean non-occluded baculoviruses.

From the above teachings, it is apparent that various modifications and variations can be made without departing from the spirit and scope of the present invention. It is therefore to be understood that this invention may be practiced otherwise than as specifically described.

Table 1. The insert size (in kilo base pair; kb) of clones in PmNOBIII *SalI* (pms) and PmNOBIII *Hin*dIII (pmh) libraries.

	PmNOB *SalI* library (pms)		PmNOB *Hin*dIII library (pmh)
clone no.	insert size (kb)	clone no.	insert size (kb)
pms1	0-0.1	pmh1	0.3
pms2	0-0.1	pmh2	7-8
pms3	0-0.1	pmh3	4
pms4	0-0.1	pmh4	0-0.1
pms5	0-0.1	pmh5	0-0.1
pms6	0-0.1	pmh6	0-0.1
pms7	4	pmh7	5
pms8	?	pmh8	3
pms9	0-0.1	pmh9	?
pms10	0-0.1	pmh10	9
pms11	0-0.1	pmh11	7-8
pms12	0-0.1	pmh12	3.5
pms13	0-0.1	pmh13	9-23
pms14	0-0.1	pmh14	2.2
pms15	0-0.1	pmh15	4
pms16	0-0.1	pmh16	6
pms17	3	pmh17	7
pms18	0-0.1	pmh18	6
pms19	?	pmh19	7
pms20	?	pmh20	1.5
pms21	0-0.1	pmh21	1.7
pms22	?	pmh22	8
pms23	0-0.1	pmh23	2.2
pms24	?	pmh24	0-0.1
pms25	?	pmh25	0-0.1
pms26	0-0.1	pmh26	0-0.1
pms27	0-0.1	pmh27	2.2
pms28	0-0.1	pmh28	6
pms29	0-0.1	pmh29	0-0.1
pms30	0-0.1	pmh30	5

	PmNOB *SalI* library (pms)		PmNOB *HindIII* library (pmh)
clone no.	insert size (kb)	clone no.	insert size (kb)
pms31	0-0.1	pmh31	4
pms32	0-0.1	pmh32	9
pms33	0-0.1	pmh33	6
pms34	0-0.1	pmh34	7-8
pms35	0-0.1	pmh35	0.5
pms36	0-0.1	pmh36	?
pms37	0-0.1	pmh37	3.5
pms38	0-0.1	pmh38	1.5
pms39	0-0.1	pmh39	4
pms40	4	pmh40	6
pms41	0-0.1	pmh41	6
pms42	0-0.1	pmh42	1.5
pms43	0-0.1	pmh43	1
pms44	?	pmh44	0-0.1
pms45	0-0.1	pmh45	6
pms46	0-0.1	pmh46	?
pms47	0-0.1	pmh47	1.7
pms48	0-0.1	pmh48	3
pms49	3	pmh49	8
pms50	?	pmh50	2.2
pms51	?	pmh51	0-0.1
pms52	2	pmh52	4
pms53	0-0.1	pmh53	0-0.1
pms54	5-6	pmh54	0-0.1
pms55	0-0.1	pmh55	8
pms56	2	pmh56	3.5
pms57	0.5	pmh57	?
pms58	0-0.1	pmh58	5
pms59	?	pmh59	3.5
pms60	4-5	pmh60	0-0.1

	PmNOB *SalI* library (pms)		PmNOB *HindIII* library (pmh)
clone no.	insert size (kb)	clone no.	insert size (kb)
pms61	?	pmh61	3.2
pms62	0-0.1	pmh62	2
pms63	0.5-1	pmh63	0-0.1
pms64	0-0.1	pmh64	4
pms65	2-3	pmh65	6
pms66	1.5	pmh66	3.5
pms67	0-0.1	pmh67	1.5
pms68	0.5	pmh68	?
pms69	0.3	pmh69	4.4
pms70	0.2	pmh70	7
pms71	?	pmh71	?
pms72	0.5	pmh72	?
pms73	?	pmh73	7
pms74	0-0.1	pmh74	?
pms75	0-0.1	pmh75	?
pms76	5-6	pmh76	?
pms77	0-0.1	pmh77	?
pms78	0.2	pmh78	0-0.1
pms79	?	pmh79	?
pms80	0.3	pmh80	?
pms81	?	pmh81	?
pms82	0.5	pmh82	?
pms83	3	pmh83	?
pms84	?	pmh84	4.4
pms85	?	pmh85	?
pms86	3	pmh86	?
pms87	?	pmh87	?
pms88	?	pmh88	?
pms89	?	pmh89	0-0.1
pms90	?	pmh90	?

	PmNOB *SalI* library (pms)		PmNOB *HindIII* library (pmh)
clone no.	insert size (kb)	clone no.	insert size (kb)
pms91	0.3	pmh91	?
pms92	6	pmh92	?
pms93	0.2	pmh93	9
pms94	9	pmh94	8
pms95	0-0.1	pmh95	?
pms96	?	pmh96	?
pms97	0.3	pmh97	?
pms98	4	pmh98	1.5
pms99	0-0.1	pmh99	?
pms100	6	pmh100	?
pms101	?	pmh101	0-0.1
pms102	0.3	pmh102	7
pms103	7-8	pmh103	1.5
pms104	?	pmh104	1
pms105	?	pmh105	9
pms106	2	pmh106	?
pms107	0-0.1	pmh107	2.2
pms108	0-0.1	pmh108	0.5
pms109	3-4	pmh109	0-0.1
pms110	3	pmh110	0-0.1
pms111	3-4	pmh111	?
pms112	1.5	pmh112	?
pms113	2	pmh113	?
pms114	?	pmh114	?
pms115	8	pmh115	2.2
pms116	2	pmh116	?
pms117	?	pmh117	?
pms118	?	pmh118	?
pms119	0-0.1	pmh119	9-23
pms120	4	pmh120	2.5

	PmNOB *SalI* library (pms)		PmNOB *HindIII* library (pmh)
clone no.	insert size (kb)	clone no.	insert size (kb)
pms121	0-0.1	pmh121	?
pms122	3	pmh122	?
pms123	0-0.1	pmh123	?
pms124	4	pmh124	?
pms125	0-0.1	pmh125	?
pms126	?	pmh126	?
pms127	?	pmh127	?
pms128	0-0.1	pmh128	?
pms129	?	pmh129	?
pms130	3	pmh130	?
pms131	0-0.1	pmh131	?
pms132	?	pmh132	?
pms133	?	pmh133	?
pms134	7-8	pmh134	?
pms135	0-0.1	pmh135	?
pms136	?	pmh136	?
pms137	?	pmh137	6
pms138	?	pmh138	2
pms139	?	pmh139	?
pms140	?	pmh140	0-0.1
pms141	2	pmh141	?
pms142	?	pmh142	4
pms143	?	pmh143	0-0.1
pms144	?	pmh144	?
pms145	?	pmh145	0-0.1
pms146	1.5	pmh146	?
pms147	?	pmh147	?
pms148	?	pmh148	?
pms149	?	pmh149	0-0.1
pms150	?	pmh150	?

	PmNOB *SalI* library (pms)		PmNOB *HindIII* library (pmh)
clone no.	insert size (kb)	clone no.	insert size (kb)
pms151	0.2	pmh151	7
pms152	?	pmh152	2.2
pms153	?	pmh153	?
pms154	?	pmh154	?
pms155	?	pmh155	?
pms156	?	pmh156	?
pms157	?	pmh157	?
pms158	?	pmh158	?
pms159	0.2	pmh159	0-0.1
pms160	1	pmh160	?
pms161	?	pmh161	?
pms162	0.3	pmh162	0-0.1
pms163	0-0.1	pmh163	0-0.1
pms164	4	pmh164	5
pms165	0-0.1	pmh165	?
pms166	0-0.1	pmh166	?
pms167	0.2	pmh167	1
pms168	1.5	pmh168	7
pms169	0-0.1	pmh169	?
pms170	0.5	pmh170	1
pms171	0-0.1	pmh171	?
pms172	0-0.1	pmh172	?
pms173	0.3	pmh173	?
pms174	0-0.1	pmh174	?
pms175	0-0.1	pmh175	1.3
pms176	0-0.1	pmh176	?
pms177	0-0.1	pmh177	?
pms178	0-0.1	pmh178	6
pms179	0-0.1	pmh179	3
pms180	1.5	pmh180	3

	PmNOB *SalI* library (pms)		PmNOB *HindIII* library (pmh)
clone no.	insert size (kb)	clone no.	insert size (kb)
pms181	4	pmh181	?
pms182	0.5	pmh182	9
pms183	0.5	pmh183	0-0.1
pms184	0-0.1	pmh184	?
pms185	0.5	pmh185	4.4
pms186	0-0.1	pmh186	0-0.1
pms187	0-0.1	pmh187	9
pms188	0-0.1	pmh188	1
pms189	2	pmh189	?
pms190	0-0.1	pmh190	?
pms191	0-0.1	pmh191	?
pms192	0-0.1	pmh192	?
pms193	0-0.1	pmh193	?
pms194	0-0.1	pmh194	?
pms195	>9	pmh195	?
pms196	0-0.1	pmh196	1.5
pms197	1.5	pmh197	?
pms198	0-0.1	pmh198	?
pms199	?	pmh199	?
pms200	?	pmh200	1.7
pms201	0-0.1	pmh201	1
pms202	?	pmh202	9
pms203	1.5	pmh203	6
pms204	?	pmh204	4.5
pms205	?	pmh205	7
pms206	?	pmh206	7
pms207	0.3	pmh207	7
pms208	?	pmh208	8
pms209	0.3	pmh209	7
pms210	0.3	pmh210	4.5

	PmNOB *Sal*I library (pms)		PmNOB *Hind*III library (pmh)
clone no.	insert size (kb)	clone no.	insert size (kb)
pms211	?	pmh211	3.2
pms212	?	pmh212	3.2
pms213	0-0.1	pmh213	4.4
pms214	1.5	pmh214	1.5
pms215	?	pmh215	3
pms216	4	pmh216	4.5
pms217	?	pmh217	3
pms218	1	pmh218	7
pms219	0-0.1	pmh219	3
pms220	?	pmh220	3.2
pms221	?	pmh221	2.2
pms222	?	pmh222	2.2
pms223	?	pmh223	2.4
pms224	?	pmh224	1.7
pms225	0.5	pmh225	4.2
pms226	?	pmh226	2.2
pms227	0.3	pmh227	4.2
pms228	0.3	pmh228	4
pms229	?	pmh229	1.5
pms230	0-0.1	pmh230	2.2
pms231	7-8	pmh231	2.2
pms232	0.3	pmh232	4.2
pms233	0.3	pmh233	2.2
pms234	0-0.1	pmh234	1.5
pms235	0.5	pmh235	4.2
pms236	0.4	pmh236	2.2
pms237	0.3	pmh237	2.2
pms238	1.5	pmh238	4.4
pms239	3	pmh239	3
pms240	1	pmh240	3

	PmNOB *SalI* library (pms)		PmNOB *HindIII* library (pmh)
clone no.	insert size (kb)	clone no.	insert size (kb)
pms241	0.5	pmh241	2.6
pms242	0.3	pmh242	5
pms243	?	pmh243	3
pms244	0-0.1	pmh244	2.5
pms245	0.5	pmh245	3.2
pms246	0-0.1	pmh246	
pms247	?	pmh247	
pms248	0.5	pmh248	
pms249	7-8	pmh249	
pms250	4-5	pmh250	
pms251	0.3	pmh251	
pms252	0-0.1	pmh252	
pms253	0.2	pmh253	
pms254	0-0.1	pmh254	
pms255	0.3	pmh255	
pms256	0.2	pmh256	
pms257	?	pmh257	
pms258	0.5	pmh258	
pms259	0.4	pmh259	
pms260	0-0.1	pmh260	
pms261	?	pmh261	
pms262	0.3	pmh262	
pms263	?	pmh263	
pms264	2.5	pmh264	
pms265	?	pmh265	
pms266	0-0.1	pmh266	
pms267	7-8	pmh267	
pms268	0.2	pmh268	
pms269	?	pmh269	
pms270	?	pmh270	

	PmNOB *SalI* library (pms)		PmNOB *HindIII* library (pmh)
clone no.	insert size (kb)	clone no.	insert size (kb)
pms271	0-0.1	pmh271	
pms272	?	pmh272	
pms273	0-0.1	pmh273	
pms274	3	pmh274	
pms275	?	pmh275	
pms276	?	pmh276	
pms277	4-5	pmh277	
pms278	0-0.1	pmh278	
pms279	0-0.1	pmh279	
pms280	0-0.1	pmh280	
pms281	?	pmh281	
pms282	?	pmh282	
pms283	0-0.1	pmh283	
pms284	?	pmh284	
pms285	0-0.1	pmh285	
pms286	0.2	pmh286	
pms287	0.2	pmh287	
pms288	?	pmh288	
pms289	4	pmh289	
pms290	0-0.1	pmh290	
pms291	3.5	pmh291	
pms292	1.5	pmh292	
pms293	3	pmh293	
pms294	0-0.1	pmh294	
pms295	1.5	pmh295	
pms296	6	pmh296	
pms297	4-5	pmh297	
pms298	?	pmh298	
pms299	?	pmh299	
pms300	0-0.1	pmh300	

	PmNOB *SalI* library (pms)		PmNOB *HindIII* library (pmh)
clone no.	insert size (kb)	clone no.	insert size (kb)
pms301	0.2	pmh301	
pms302	0.3	pmh302	
pms303	0.3	pmh303	
pms304	0-0.1	pmh304	
pms305	0-0.1	pmh305	
pms306	0.3	pmh306	
pms307	?	pmh307	
pms308	?	pmh308	
pms309	0.5	pmh309	
pms310	0-0.1	pmh310	
pms311	0.5	pmh311	
pms312	6	pmh312	
pms313	3	pmh313	
pms314	?	pmh314	
pms315	?	pmh315	
pms316	0-0.1	pmh316	
pms317	?	pmh317	
pms318	?	pmh318	
pms319	0-0.1	pmh319	
pms320	?	pmh320	
pms321	4-5	pmh321	
pms322	0.5	pmh322	
pms323	?	pmh323	
pms324	4	pmh324	
pms325	0.2	pmh325	
pms326	2	pmh326	
pms327	1	pmh327	
pms328	0-0.1	pmh328	
pms329	?	pmh329	
pms330	?	pmh330	

	PmNOB *SalI* library (pms)		PmNOB *HindIII* library (pmh)
clone no.	insert size (kb)	clone no.	insert size (kb)
pms331	4-5	pmh331	
pms332	0.2	pmh332	
pms333	?	pmh333	
pms334	2	pmh334	
pms335	0-0.1	pmh335	
pms336	0.2	pmh336	
pms337	0-0.1	pmh337	
pms338	?	pmh338	
pms339	0-0.1	pmh339	
pms340	?	pmh340	
pms341	0-0.1	pmh341	
pms342	?	pmh342	
pms343	0-0.1	pmh343	
pms344	?	pmh344	
pms345	2	pmh345	
pms346	0-0.1	pmh346	
pms347	0-0.1	pmh347	
pms348	0.2	pmh348	
pms349	0.5	pmh349	
pms350	0-0.1	pmh350	
pms351	4	pmh351	
pms352	0-0.1	pmh352	
pms353	0.3	pmh353	
pms354	0-0.1	pmh354	
pms355	0-0.1	pmh355	
pms356	0-0.1	pmh356	
pms357	0-0.1	pmh357	
pms358	0-0.1	pmh358	
pms359	0-0.1	pmh359	
pms360	?	pmh360	

	PmNOB *SalI* library (pms)		PmNOB *HindIII* library (pmh)
clone no.	insert size (kb)	clone no.	insert size (kb)
pms361	0-0.1	pms361	
pms362	?	pms362	
pms363	?	pms363	
pms364	?	pms364	
pms365	?	pmh365	
pms366	0-0.1	pmh366	
pms367	?	pmh367	
pms368	?	pmh368	
pms369	0-0.1	pmh369	
pms370	?	pmh370	
pms371	0.5	pmh371	
pms372	0.3	pmh372	
pms373	0.5	pmh373	
pms374	?	pmh374	
pms375	?	pmh375	
pms376	3	pmh376	
pms377	?	pmh377	
pms378	6	pmh378	
pms379	?	pmh379	
pms380	?	pmh380	
pms381	4	pmh381	
pms382	?	pmh382	
pms383	?	pmh383	
pms384	?	pmh384	
pms385	0-0.1	pmh385	
pms386	?	pmh386	
pms387	?	pmh387	
pms388	?	pmh388	
pms389	?	pmh389	
pms390	?	pmh390	

	PmNOB *SalI* library (pms)		PmNOB *HindIII* library (pmh)
clone no.	insert size (kb)	clone no.	insert size (kb)
pms391	0-0.1	pmh391	
pms392	?	pmh392	
pms393	?	pmh393	
pms394	?	pmh394	
pms395	0-0.1	pmh395	
pms396	0-0.1	pmh396	
pms397	0-0.1	pmh397	
pms398	?	pmh398	
pms399	?	pmh399	
pms400	0-0.1	pmh400	
pms401	?	pmh401	
pms402	0.2	pmh402	
pms403	0-0.1	pmh403	
pms404	0-0.1	pmh404	
pms405	0-0.1	pmh405	
pms406	0-0.1	pmh406	
pms407	?	pmh407	
pms408	0.5	pmh408	
pms409	?	pmh409	
pms410	0.5	pmh410	
pms411	0-0.1	pmh411	
pms412	1	pmh412	
pms413	0.2	pmh413	
pms414	4.5	pmh414	
pms415	6	pmh415	
pms416	0-0.1	pmh416	
pms417	0-0.1	pmh417	
pms418	4	pmh418	
pms419	0-0.1	pmh419	?
pms420	2	pmh420	1.7

	PmNOB *SalI* library (pms)		PmNOB *HindIII* library (pmh)
clone no.	insert size (kb)	clone no.	insert size (kb)
pms421	0.2	pmh421	6
pms422	0.5	pmh422	9
pms423	0-0.1	pmh423	?
pms424	0-0.1	pmh424	0-0.1
pms425	1.2	pmh425	0-0.1
pms426	?	pmh426	0-0.1
pms427	4.5	pmh427	4
pms428	0-0.1	pmh428	7
pms429	6	pmh429	?
pms430	0.3	pmh430	3.5
pms431	0.5	pmh431	8
pms432	0-0.1	pmh432	1.5
pms433	0.2	pmh433	4
pms434	0-0.1	pmh434	?
pms435	0-0.1	pmh435	7
pms436	0.2	pmh436	4.4
pms437	0-0.1	pmh437	0-0.1
pms438	0-0.1	pmh438	6
pms439	0.5	pmh439	5
pms440	0-0.1	pmh440	?
pms441	2	pmh441	7
pms442	?	pmh442	?
pms443	2.5	pmh443	?
pms444	4.5	pmh444	0-0.1
pms445	0.2	pmh445	2
pms446	4.5	pmh446	?
pms447	3	pmh447	9-23
pms448	0-0.1	pmh448	1.5
pms449	0-0.1	pmh449	3.2
pms450	0-0.1	pmh450	0-0.1

	PmNOB *SalI* library (pms)		PmNOB *HindIII* library (pmh)
clone no.	insert size (kb)	clone no.	insert size (kb)
pms451	0-0.1	pmh451	0-0.1
pms452	0.5	pmh452	?
pms453	0-0.1	pmh453	4.4
pms454	0.3	pmh454	1
pms455	0-0.1	pmh455	1
pms456	0-0.1	pmh456	0-0.1
pms457	0-0.1	pmh457	?
pms458	3	pmh458	?
pms459	0.5	pmh459	?
pms460	4-5	pmh460	?
pms461	0-0.1	pmh461	?
pms462	3-4	pmh462	?
pms463	0-0.1	pmh463	?
pms464	0-0.1	pmh464	?
pms465	0-0.1	pmh465	?
pms466	5	pmh466	2.6
pms467	0-0.1	pmh467	?
pms468	6	pmh468	4.2
pms469	0-0.1	pmh469	2.4
pms470	5	pmh470	4
pms471	9	pmh471	4.4
pms472	0-0.1	pmh472	8
pms473	9-23	pmh473	3
pms474	0-0.1	pmh474	4
pms475	0.5	pmh475	4.7
pms476	0-0.1	pmh476	?
pms477	5	pmh477	?
pms478	0-0.1	pmh478	?
pms479	0-0.1	pmh479	?
pms480	0.3	pmh480	2

	PmNOB *Sal*I library (pms)		PmNOB *Hin*dIII library (pmh)
clone no.	insert size (kb)	clone no.	insert size (kb)
pms481	1	pmh481	?
pms482	5	pmh482	0-0.1
pms483	1.5	pmh483	?
pms484	9	pmh484	2.2
pms485	0.2	pmh485	1.5
pms486	0-0.1	pmh486	2.2
pms487	0-0.1	pmh487	?
pms488	1.5	pmh488	2.2
pms489	5	pmh489	?
pms490	0-0.1	pmh490	4
pms491	1.5	pmh491	1.5
pms492	0-0.1	pmh492	4.8
pms493	0-0.1	pmh493	?
pms494	9	pmh494	?
pms495	0.2	pmh495	?
pms496	0-0.1	pmh496	0-0.1
pms497	1.5	pmh497	0.5
pms498	0-0.1	pmh498	?
pms499	0.3	pmh499	7
pms500	0.5	pmh500	0-0.1
pms501	0-0.1	pmh501	?
pms502	0-0.1	pmh502	2.6
pms503	0-0.1	pmh503	1.8
pms504	0-0.1	pmh504	0.5
pms505	0-0.1	pmh505	2.2
pms506	3	pmh506	?
pms507	0.3	pmh507	1.8
pms508	0.2	pmh508	?
pms509	3	pmh509	0.7
pms510	5	pmh510	2.3

	PmNOB *SalI* library (pms)		PmNOB *HindIII* library (pmh)
clone no.	insert size (kb)	clone no.	insert size (kb)
pms511	0-0.1	pmh511	?
pms512	0.5	pmh512	9-23
pms513	0-0.1	pmh513	1.5
pms514	4	pmh514	?
pms515	0-0.1	pmh515	3.2
pms516	6	pmh516	8
pms517	0-0.1	pmh517	?
pms518	0-0.1	pmh518	0-0.1
pms519	0.2	pmh519	3.5
pms520	0-0.1	pmh520	4.4
pms521	0.3	pmh521	?
pms522	5	pmh522	5
pms523	0.6	pmh523	4.4
pms524	0.3	pmh524	7
pms525	0-0.1	pmh525	1.5
pms526	6	pmh526	5.5
pms527	0-0.1	pmh527	1.5
pms528	6	pmh528	7
pms529	0.2	pmh529	4.4
pms530	3	pmh530	7
pms531	3	pmh531	2.2
pms532	0-0.1	pmh532	8
pms533	0-0.1	pmh533	9
pms534	5	pmh534	1.5
pms535	0-0.1	pmh535	?
pms536	5	pmh536	1.5
pms537	0-0.1	pmh537	?
pms538	2	pmh538	?
pms539	0-0.1	pmh539	2.2
pms540	0-0.1	pmh540	?

	PmNOB *SalI* library (pms)		PmNOB *HindIII* library (pmh)
clone no.	insert size (kb)	clone no.	insert size (kb)
pms541	0-0.1	pmh541	?
pms542	0-0.1	pmh542	4.6
pms543	3	pmh543	0-0.1
pms544	4	pmh544	1.5
pms545	0-0.1	pmh545	8
pms546	0-0.1	pmh546	9
pms547	0-0.1	pmh547	1.5
pms548	1.5	pmh548	0.2
pms549	3	pmh549	0.5
pms550	4.4	pmh550	?
pms551	0-0.1	pmh551	0-0.1
pms552	0-0.1	pmh552	5.5
pms553	0-0.1	pmh553	3
pms554	0-0.1	pmh554	0-0.1
pms555	4.4	pmh555	7
pms556	0-0.1	pmh556	?
pms557	0-0.1	pmh557	?
pms558	3	pmh558	2.6
pms559	0-0.1	pmh559	?
pms560	0.5	pmh560	?
pms561	0-0.1	pmh561	0-0.1
pms562	2	pmh562	4
pms563	0-0.1	pmh563	2.2
pms564	0-0.1	pmh564	?
pms565	0-0.1	pmh565	?
pms566	6	pmh566	?
pms567	9	pmh567	?
pms568	0.3	pmh568	?
pms569	0.5	pmh569	?
pms570	0.2	pmh570	5

	PmNOB *SalI* library (pms)		PmNOB *HindIII* library (pmh)
clone no.	insert size (kb)	clone no.	insert size (kb)
pms571	0.5	pmh571	4
pms572	0-0.1	pmh572	?
pms573	0-0.1	pmh573	?
pms574	0.2	pmh574	7
pms575	0.3	pmh575	5.5
pms576	3	pmh576	8
pms577	0.5	pmh577	9
pms578	0-0.1	pmh578	0-0.1
pms579	0-0.1	pmh579	9
pms580	0-0.1	pmh580	0-0.1
pms581	?	pmh581	?
pms582	?	pmh582	0-0.1
pms583	?	pmh583	5
pms584	?	pmh584	4
pms585	?		
pms586	?		
pms587	?		
pms588	?		
pms589	?		
pms590	0-0.1		
pms591	0-0.1		
pms592	?		

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Guang-Hsiung Kou

Chung-Hsiung Wang

Chu-Fang Lo

(ii) TITLE OF INVENTION: Identification, Purification and Detection of WSBV (Baculovirus Associated with White Spot Syndrome)

(iii) NUMBER OF SEQUENCES: 13

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE:

(B) STREET:

(C) CITY:

(D) STATE:

(E) COUNTRY:

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE:Floppy disk-3.5 inch, 1.44M storage

(B) COMPUTER: IBM PC/386 Compatible

(C) OPERATION SYSTEM: MS-DOS 5.0

(D) SOFTWARE: PE2

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: none

(B) FILING DATE: new application

(C) CLASSIFICATION: none

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: none

(B) FILING DATE: none

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME:

(B) REGISTRATION NUMBER:

(C) REFERENCE/DOCKET NUMBER:

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE:

(B) TELEFAX:

(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1461 bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double stranded (only one stand shown)

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: WSBV (Baculovirus Associated with White Spot Syndrome)

(B) STRAIN: PmNOB II

(vii) IMMEDIATE SOURCE:

(A) LIBRARY:

(B) CLONE:

(ix) FEATURE:

(A) NAME/KEY:

(B) LOCATION:

(ix) FEATURE:

(A) NAME/KEY:

(B) LOCATION:

(ix) FEATURE:

(A) NAME/KEY:

(B) LOCATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1

GTCGACAGAC TACTAACTTC AGCCTATCTA GTAAAACAAG CTAAAAGATT CGACGGAGTT 60

GACCCAGCCT TCCCTGCCGC CCTCACCTGC GCTTCTCACC TCATGCTTTC TTCCATGGAT 120

TCCCATACAA AGTCATCTTT CATGGACAAC ATCAAATTGC ACATGACTGA TACTCAATGC 180

TTCTTCAAGA ACATTGAACG ATTTGAGAAA TTCTTGGGAA GATATGGGGA CGAATACGCC 240

ATGTCCCACA AGCAAAATTG TAACTGCCCC TTCCATCTCC ACCACACTTT TACTCCCTCA 300

GATAACGAGC ATCTGGTATC CTCTTTCGCA TTCGCCCGCC CAGAAGTCTC CATGGAAGAA 360

ATTAGAGCCA CACCCTATCA GGCCAACAAG CTTATTAGTG ACAAACATTA CGTGATGAAC 420

ATGTCCAAGA TCGATTCTAG AGTAACAGGA TCTTCCCTCC TTAAGAAGGT TAGCGAATGG 480

ACTGAAATGA GAATGAACTC CAACTTTAAT GGAACATTTG AACCATCAAG ACTCGCCCTC 540

TCCAACTCTG GCATGACAAC GGCAGGAGTC AACCTCGACG TTATTGTCAA ACCAAATAAT 600

GCAAGAAGTG TACTAGGAAT ATTGGAATGT CATCGCCAGC ACGTGTGCAC CGCCGACGCC 660

AAGGGAACTG TCGCTTCAGC CATGCCAGCC GTCTTCCAGG CAACCGATGG AAACGGTAAC 720

GAATCTGAAC TGATCCAGAA TGCTCTGCCA AGGAACAGAT ACATCCAAAA GAGCACAATG 780

AACGCTCAAA CTGTCGTGTT TGCTAATGTT TTGGAACAAC TTATCGCCGA TCTTGGAAAG 840

GTTATCGTGA ACGAACTGGC CGGCACCATC GCTGAATCTG TACCAGAAAG CGTATATGAA 900

AACACCAAGG AAATGATTGA TAGACTAGGC TCTGACGACC TCTTCAAATC TAATAATAAT 960

GGAGGAGTAG AATCAATGGA TTATGAAGAT AGCGAAACAA CATCCAACAA TGGTCCCGTC 1020

CTCATCTCAG AAGCCATGAA GAATGCCGTC TATCACACAC TAATTTCCGG CAAGGCAGCT 1080

CGCCCGGAAA ATGTACCATT CGCCTCATGC GCCAGCGGCC CTCTCGCCTT TGATTTCCTT 1140

CTGTCAAAGG GAGATACATT CGAAGAAAAG AACGCCGAAC AAGGTGCAGC AGCTGCCGTA 1200

TCCTCTACCT ATTCTTCCTC TTCTAACACT ACTCTTCGTA AGCATTTGGC TCGAGTTTTC 1260

GAAGCCATCT CTAAGCAAGT AACTGATGCT GAATTCAAGG ATATCCTCAA CGATATCGAA 1320

CGTAATATTT CTTCTGACTA TACTAACTGT CCACCAAATA CTAACCAAAA TGCCTTTGCT 1380

CTAGCTATCA AGAGAGAATT CAGCAGAATT GTTTCCTTCT TAACCATTCT TCGTAAGAAC 1440

ATTACACCCG CATTAGTCGA C

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1447 bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double stranded (only one stand shown)

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: WSBV (Baculovirus Associated with White Spot Syndrome)

(B) STRAIN: PmNOB II

(vii) IMMEDIATE SOURCE:

(A) LIBRARY:

(B) CLONE:

(ix) FEATURE:

(A) NAME/KEY:

(B) LOCATION:

(ix) FEATURE:

(A) NAME/KEY:

(B) LOCATION:

(ix) FEATURE:

(A) NAME/KEY:

(B) LOCATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2

ACTACTAACT TCAGCCTATC TAGTAAAACA AGCTAAAAGA TTCGACGGAG TTGACCCAGC 60

CTTCCCTGCC GCCCTCACCT GCGCTTCTCA CCTCATGCTT TCTTCCATGG ATTCCCATAC 120

AAAGTCATCT TTCATGGACA ACATCAAATT GCACATGACT GATACTCAAT GCTTCTTCAA 180

GAACATTGAA CGATTTGAGA AATTCTTGGG AAGATATGGG GACGAATACG CCATGTCCCA 240

CAAGCAAAAT TGTAACTGCC CCTTCCATCT CCACCACACT TTTACTCCCT CAGATAACGA 300

GCATCTGGTA TCCTCTTTCG CATTCGCCCG CCCAGAAGTC TCCATGGAAG AAATTAGAGC 360

CACACCCTAT CAGGCCAACA AGCTTATTAG TGACAAACAT TACGTGATGA ACATGTCCAA 420

GATCGATTCT AGAGTAACAG GATCTTCCCT CCTTAAGAAG GTTAGCGAAT GGACTGAAAT 480

GAGAATGAAC TCCAACTTTA ATGGAACATT TGAACCATCA AGACTCGCCC TCTCCAACTC 540

TGGCATGACA ACGGCAGGAG TCAACCTCGA CGTTATTGTC AAACCAAATA ATGCAAGAAG 600

TGTACTAGGA ATATTGGAAT GTCATCGCCA GCACGTGTGC ACCGCCGACG CCAAGGGAAC 660

TGTCGCTTCA GCCATGCCAG CCGTCTTCCA GGCAACCGAT GGAAACGGTA ACGAATCTGA 720

ACTGATCCAG AATGCTCTGC CAAGGAACAG ATACATCCAA AAGAGCACAA TGAACGCTCA 780

AACTGTCGTG TTTGCTAATG TTTTGGAACA ACTTATCGCC GATCTTGGAA AGGTTATCGT 840

GAACGAACTG GCCGGCACCA TCGCTGAATC TGTACCAGAA AGCGTATATG AAAACACCAA 900

GGAAATGATT GATAGACTAG GCTCTGACGA CCTCTTCAAA TCTAATAATA ATGGAGGAGT 960

AGAATCAATG GATTATGAAG ATAGCGAAAC AACATCCAAC AATGGTCCCG TCCTCATCTC 1020

AGAAGCCATG AAGAATGCCG TCTATCACAC ACTAATTTCC GGCAAGGCAG CTCGCCCGGA 1080

AAATGTACCA TTCGCCTCAT GCGCCAGCGG CCCTCTCGCC TTTGATTTCC TTCTGTCAAA 1140

GGGAGATACA TTCGAAGAAA AGAACGCCGA ACAAGGTGCA GCAGCTGCCG TATCCTCTAC 1200

CTATTCTTCC TCTTCTAACA CTACTCTTCG TAAGCATTTG GCTCGAGTTT TCGAAGCCAT 1260

CTCTAAGCAA GTAACTGATG CTGAATTCAA GGATATCCTC AACGATATCG AACGTAATAT 1320

TTCTTCTGAC TATACTAACT GTCCACCAAA TACTAACCAA AATGCCTTTG CTCTAGCTAT 1380

CAAGAGAGAA TTCAGCAGAA TTGTTTCCTT CTTAACCATT CTTCGTAAGA ACATTACACC 1440

CGCATTA

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single stranded (from 5' to 3')

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide probe

(iv) ANTI-SENSE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3

ACTACTAACT TCAGCCTATC TAG

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single stranded (from 5' to 3')

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide probe

(iv) ANTI-SENSE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4

TAATGCGGGT GTAATGTTCT TACGA

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single stranded (from 5' to 3')

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide probe

(iv) ANTI-SENSE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5

GTAACTGCCC CTTCCATCTC CA

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single stranded (from 5' to 3')

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide probe

(iv) ANTI-SENSE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6

TACGGCAGCT GCTGCACCTT GT

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single stranded (from 5' to 3')

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide probe

(iv) ANTI-SENSE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7

TGGGAAGATA TGGGGACGAA T

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single stranded (from 5' to 3')

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide probe

(iv) ANTI-SENSE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8

CGAAGAGTAG TGTTAGAAGA GGA

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single stranded (from 5' to 3')

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide probe

(iv) ANTI-SENSE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9

AGAAGGTTAG CGAATGGACT G

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single stranded (from 5' to 3')

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide probe

(iv) ANTI-SENSE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10

TTGAAGAGGT CGTCAGAGCC T

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single stranded (from 5' to 3')

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide probe

(iv) ANTI-SENSE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11

GAAACGGTAA CGAATCTGAA CTG

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single stranded (from 5' to 3')

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide probe

(iv) ANTI-SENSE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12

CAGTCCATTC GCTAACCT

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bp

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single stranded (from 5' to 3')

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide probe

(iv) ANTI-SENSE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13

CGTCCCCATA TCTTCCCA

Claims

1. A subtantially pure viral isolate of baculovirus associated with white spot syndrome (WSBV).

2. A WSBV according to Claim 1, wherein the virus comprises a genome having the restriction enzyme fragments profile as shown in Figure 11.

3. A WSBV according to Claim 1 or 2, wherein the gemone of the virus comprises a SalI DNA fragment having a nucleotide sequence as shown below:

```
1    GTCGA CAGAC TACTA ACTTC AGCCT ATCTA GTAAA ACAAG CTAAA AGATT
51   CGACG GAGTT GACCC AGCCT TCCCT GCCGC CCTCA CCTGC GCTTC TCACC
101  TCATG CTTTC TTCCA TGGAT TCCCA TACAA AGTCA TCTTT CATGG ACAAC
151  ATCAA ATTGC ACATG ACTGA TACTC AATGC TTCTT CAAGA ACATT GAACG
201  ATTTG AGAAA TTCTT GGGAA GATAT GGGGA CGAAT ACGCC ATGTC CCACA
251  AGCAA AATTG TAACT GCCCC TTCCA TCTCC ACCAC ACTTT TACTC CCTCA
301  GATAA CGAGC ATCTG GTATC CTCTT TCGCA TTCGC CCGCC CAGAA GTCTC
351  CATGG AAGAA ATTAG AGCCA CACCC TATCA GGCCA ACAAG CTTAT TAGTG
401  ACAAA CATTA CGTGA TGAAC ATGTC CAAGA TCGAT TCTAG AGTAA CAGGA
451  TCTTC CCTCC TTAAG AAGGT TAGCG AATGG ACTGA AATGA GAATG AACTC
501  CAACT TTAAT GGAAC ATTTG AACCA TCAAG ACTCG CCCTC TCCAA CTCTG
551  GCATG ACAAC GGCAG GAGTC AACCT CGACG TTATT GTCAA ACCAA ATAAT
601  GCAAG AAGTG TACTA GGAAT ATTGG AATGT CATCG CCAGC ACGTG TGCAC
651  CGCCG ACGCC AAGGG AACTG TCGCT TCAGC CATGC CAGCC GTCTT CCAGG
701  CAACC GATGG AAACG GTAAC GAATC TGAAC TGATC CAGAA TGCTC TGCCA
751  AGGAA CAGAT ACATC CAAAA GAGCA CAATG AACGC TCAAA CTGTC GTGTT
801  TGCTA ATGTT TTGGA ACAAC TTATC GCCGA TCTTG GAAAG GTTAT CGTGA
851  ACGAA CTGGC CGGCA CCATC GCTGA ATCTG TACCA GAAAG CGTAT ATGAA
901  AACAC CAAGG AAATG ATTGA TAGAC TAGGC TCTGA CGACC TCTTC AAATC
951  TAATA ATAAT GGAGG AGTAG AATCA ATGGA TTATG AAGAT AGCGA AACAA
1001 CATCC AACAA TGGTC CCGTC CTCAT CTCAG AAGCC ATGAA GAATG CCGTC
1051 TATCA CACAC TAATT TCCGG CAAGG CAGCT CGCCC GGAAA ATGTA CCATT
1101 CGCCT CATGC GCCAG CGGCC CTCTC GCCTT TGATT TCCTT CTGTC AAAGG
1151 GAGAT ACATT CGAAG AAAAG AACGC CGAAC AAGGT GCAGC AGCTG CCGTA
1201 TCCTC TACCT ATTCT TCCTC TTCTA ACACT ACTCT TCGTA AGCAT TTGGC
1251 TCGAG TTTTC GAAGC CATCT CTAAG CAAGT AACTG ATGCT GAATT CAAGG
1301 ATATC CTCAA CGATA TCGAA CGTAA TATTT CTTCT GACTA TACTA ACTGT
1351 CCACC AAATA CTAAC CAAAA TGCCT TTGCT CTAGC TATCA AGAGA GAATT
1401 CAGCA GAATT GTTTC CTTCT TAACC ATTCT TCGTA AGAAC ATTAC ACCCG
1451 CATTA GTCGA C
```

4. A WSBV according to Claim 1, wherein the gemone of the virus comprises a SalI DNA fragment having a partial nucleotide sequence from the 9th nucleotide to the 1455th nucleotide of the nucleotide sequence shown in Claim 1.

5. A WSBV according to Claim 1, wherein the virus is PmNOBIII or PmNOBIII related agents.

6. A method for obtaining a high quantity of substantially pure non-occluded baculovirus (NOB) from a host organism infected thereby, comprising the steps of:

   a) obtaining a sample from a host organism infected with a NOB virus;
   b) treating the sample with protease inhibitors in an amount sufficient to inhibit the degradation of NOB virus; and
   c) purifying the virus.

7. A method according to Claim 6, wherein the purification step (c) is performed by centrifugation.

8. A method according to Claim 7, wherein the purification step (c) is performed by sucrose gredient centrifugation.

9. A method according to Claim 7, wherein the protease inhibitors are removed post centrifugation.

10. A method according to Claim 6, wherein the virus is WSBV.

11. A method according to Claim 6, wherein the virus is PmNOBIII or PmNOBIII related agents.

12. A polynucleotide comprising

(i) a nucleotide sequence as shown below:

```
1    GTCGA CAGAC TACTA ACTTC AGCCT ATCTA GTAAA ACAAG CTAAA AGATT
51   CGACG GAGTT GACCC AGCCT TCCCT GCCGC CCTCA CCTGC GCTTC TCACC
101  TCATG CTTTC TTCCA TGGAT TCCCA TACAA AGTCA TCTTT CATGG ACAAC
151  ATCAA ATTGC ACATG ACTGA TACTC AATGC TTCTT CAAGA ACATT GAACG
201  ATTTG AGAAA TTCTT GGGAA GATAT GGGGA CGAAT ACGCC ATGTC CCACA
251  AGCAA AATTG TAACT GCCCC TTCCA TCTCC ACCAC ACTTT TACTC CCTCA
301  GATAA CGAGC ATCTG GTATC CTCTT TCGCA TTCGC CCGCC CAGAA GTCTC
351  CATGG AAGAA ATTAG AGCCA CACCC TATCA GGCCA ACAAG CTTAT TAGTG
401  ACAAA CATTA CGTGA TGAAC ATGTC CAAGA TCGAT TCTAG AGTAA CAGGA
451  TCTTC CCTCC TTAAG AAGGT TAGCG AATGG ACTGA AATGA GAATG AACTC
501  CAACT TTAAT GGAAC ATTTG AACCA TCAAG ACTCG CCCTC TCCAA CTCTG
551  GCATG ACAAC GGCAG GAGTC AACCT CGACG TTATT GTCAA ACCAA ATAAT
601  GCAAG AAGTG TACTA GGAAT ATTGG AATGT CATCG CCAGC ACGTG TGCAC
651  CGCCG ACGCC AAGGG AACTG TCGCT TCAGC CATGC CAGCC GTCTT CCAGG
701  CAACC GATGG AAACG GTAAC GAATC TGAAC TGATC CAGAA TGCTC TGCCA
751  AGGAA CAGAT ACATC CAAAA GAGCA CAATG AACGC TCAAA CTGTC GTGTT
801  TGCTA ATGTT TTGGA ACAAC TTATC GCCGA TCTTG GAAAG GTTAT CGTGA
851  ACGAA CTGGC CGGCA CCATC GCTGA ATCTG TACCA GAAAG CGTAT ATGAA
901  AACAC CAAGG AAATG ATTGA TAGAC TAGGC TCTGA CGACC TCTTC AAATC
951  TAATA ATAAT GGAGG AGTAG AATCA ATGGA TTATG AAGAT AGCGA AACAA
1001 CATCC AACAA TGGTC CCGTC CTCAT CTCAG AAGCC ATGAA GAATG CCGTC
1051 TATCA CACAC TAATT TCCGG CAAGG CAGCT CGCCC GGAAA ATGTA CCATT
1101 CGCCT CATGC GCCAG CGGCC CTCTC GCCTT TGATT TCCTT CTGTC AAAGG
1151 GAGAT ACATT CGAAG AAAAG AACGC CGAAC AAGGT GCAGC AGCTG CCGTA
1201 TCCTC TACCT ATTCT TCCTC TTCTA ACACT ACTCT TCGTA AGCAT TTGGC
1251 TCGAG TTTTC GAAGC CATCT CTAAG CAAGT AACTG ATGCT GAATT CAAGG
1301 ATATC CTCAA CGATA TCGAA CGTAA TATTT CTTCT GACTA TACTA ACTGT
1351 CCACC AAATA CTAAC CAAAA TGCCT TTGCT CTAGC TATCA AGAGA GAATT
1401 CAGCA GAATT GTTTC CTTCT TAACC ATTCT TCGTA AGAAC ATTAC ACCCG
1451 CATTA GTCGA C
```

(ii) a nucleotide sequence complementary to the nucleotide sequence of (i); or
(iii) a nucleotide sequence hybridizable to the nucleotide sequence of (i) or (ii).

13. A nucleotide segment derived from a polynucleotide as defined in Claim 12, said nucleotide segment being capable of hybridizing to a genomic DNA of WSBV.

14. A nucleotide segment according to Claim 13, wherein the nucleotide segment is labled with a reporter digoxigenin (DIG).

15. A nucleotide segment derived from a polynucleotide as defined in Claim 12, said nucleotide s gment being useful as a primer in the PCR amplification of a genomic DNA of WSBV.

16. A nucleotide segment according to Claim 15, wherein the nucleotide segment is labeled with a reporter digoxigenin (DIG).

17. A nucleotide segment as defined in any one of Claims 13 to 16, comprising (146F1, 146R1, 146F2 or 146R2).

18. A method for detecting WSBV viral infection in shrimps, comprising the steps of:

a) taking a sample from a leg or hemolymph of a live shrimp;
b) detecting the presence of WSBV in said sample by

i) southern hybridization with a nucleotide segement according to Claim 13;
ii) dot bloting with a nucleotide segement according to Claim 13; or
iii) PCR amplification reaction with use of a pair of primers as defined in Claim 15.

19. A method according to Claim 16, wherein the nucleotide is labled with a reporter digoxigenin (DIG) for dot bloting.

20. A polynucleotide comprising

(i) a nucleotide sequence from the 9th nucleotide to the 1455th nucleotide sequence of the following sequenc :

```
1     GTCGA CAGAC TACTA ACTTC AGCCT ATCTA GTAAA ACAAG CTAAA AGATT
51    CGACG GAGTT GACCC AGCCT TCCCT GCCGC CCTCA CCTGC GCTTC TCACC
101   TCATG CTTTC TTCCA TGGAT TCCCA TACAA AGTCA TCTTT CATGG ACAAC
151   ATCAA ATTGC ACATG ACTGA TACTC AATGC TTCTT CAAGA ACATT GAACG
201   ATTTG AGAAA TTCTT GGGAA GATAT GGGGA CGAAT ACGCC ATGTC CCACA
251   AGCAA AATTG TAACT GCCCC TTCCA TCTCC ACCAC ACTTT TACTC CCTCA
301   GATAA CGAGC ATCTG GTATC CTCTT TCGCA TTCGC CCGCC CAGAA GTCTC
351   CATGG AAGAA ATTAG AGCCA CACCC TATCA GGCCA ACAAG CTTAT TAGTG
401   ACAAA CATTA CGTGA TGAAC ATGTC CAAGA TCGAT TCTAG AGTAA CAGGA
451   TCTTC CCTCC TTAAG AAGGT TAGCG AATGG ACTGA AATGA GAATG AACTC
501   CAACT TTAAT GGAAC ATTTG AACCA TCAAG ACTCG CCCTC TCCAA CTCTG
551   GCATG ACAAC GGCAG GAGTC AACCT CGACG TTATT GTCAA ACCAA ATAAT
601   GCAAG AAGTG TACTA GGAAT ATTGG AATGT CATCG CCAGC ACGTG TGCAC
651   CGCCG ACGCC AAGGG AACTG TCGCT TCAGC CATGC CAGCC GTCTT CCAGG
701   CAACC GATGG AAACG GTAAC GAATC TGAAC TGATC CAGAA TGCTC TGCCA
751   AGGAA CAGAT ACATC CAAAA GAGCA CAATG AACGC TCAAA CTGTC GTGTT
801   TGCTA ATGTT TTGGA ACAAC TTATC GCCGA TCTTG GAAAG GTTAT CGTGA
851   ACGAA CTGGC CGGCA CCATC GCTGA ATCTG TACCA GAAAG CGTAT ATGAA
901   AACAC CAAGG AAATG ATTGA TAGAC TAGGC TCTGA CGACC TCTTC AAATC
951   TAATA ATAAT GGAGG AGTAG AATCA ATGGA TTATG AAGAT AGCGA AACAA
1001  CATCC AACAA TGGTC CCGTC CTCAT CTCAG AAGCC ATGAA GAATG CCGTC
1051  TATCA CACAC TAATT TCCGG CAAGG CAGCT CGCCC GGAAA ATGTA CCATT
1101  CGCCT CATGC GCCAG CGGCC CTCTC GCCTT TGATT TCCTT CTGTC AAAGG
1151  GAGAT ACATT CGAAG AAAAG AACGC CGAAC AAGGT GCAGC AGCTG CCGTA
1201  TCCTC TACCT ATTCT TCCTC TTCTA ACACT ACTCT TCGTA AGCAT TTGGC
1251  TCGAG TTTTC GAAGC CATCT CTAAG CAAGT AACTG ATGCT GAATT CAAGG
1301  ATATC CTCAA CGATA TCGAA CGTAA TATTT CTTCT GACTA TACTA ACTGT
1351  CCACC AAATA CTAAC CAAAA TGCCT TTGCT CTAGC TATCA AGAGA GAATT
1401  CAGCA GAATT GTTTC CTTCT TAACC ATTCT TCGTA AGAAC ATTAC ACCCG
1451  CATTA GTCGA C
```

(ii) a nucleotide sequence complementary to the nucleotide sequence of (i); or
(iii) a nucleotide sequence hybridizable to the nucleotide sequence of (i) or (ii).

21. A nucleotide segment derived from a polynucleotide as defined in Claim 20, said nucleotide segment being capable of hybridizing to a genomic DNA of WSBV.

22. A nucleotide segment according to Claim 21, wherein the nucleotide segment is labled with a reporter digoxigenin (DIG).

23. A nucleotide segment derived from a polynucleotide as defined in Claim 22, said nucleotide segment being useful as a primer in the PCR amplification of a genomic DNA of WSBV.

24. A nucleotide segment according to Claim 23, wherein the nucleotide segment is labled with a reporter digoxigenin (DIG).

25. A nucleotide segment as defined in any one of Claims 21 to 24, comprising (146F1, 146R1, 146F2 or 146R2).

26. A method for detecting WSBV viral infection in shrimps, comprising the steps of:

a) taking a sample from a leg or hemolymph of a live shrimp;
b) detecting the presence of WSBV in said sample by

i) southern hybridization with a nucleotide segement according to Claim 21;
ii) dot bloting with a nucleotide segement according to Claim 21; or
iii) PCR amplification reaction with use of a pair of primers as defined in Claim 23.

27. A method according to Claim 26, wherein the nucleotide is labled with a reporter digoxigenin (DIG) for dot bloting.

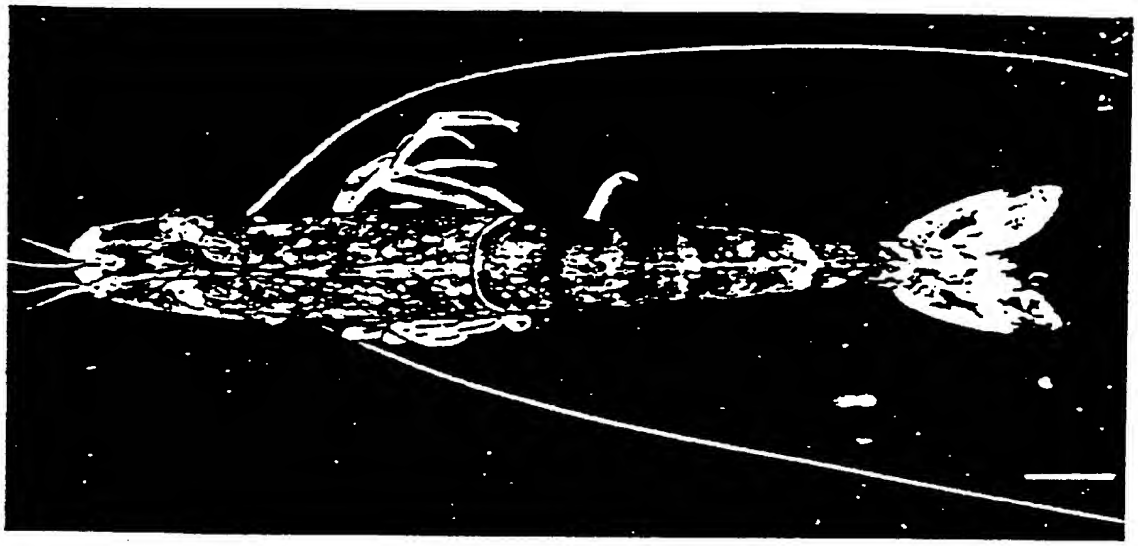

FIG.1

FIG.2

FIG.3

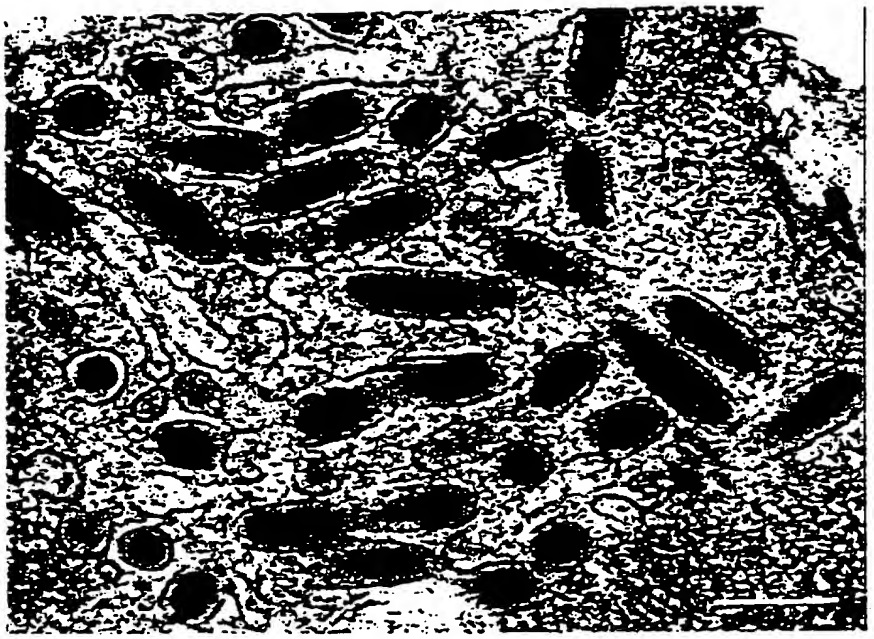

FIG.4

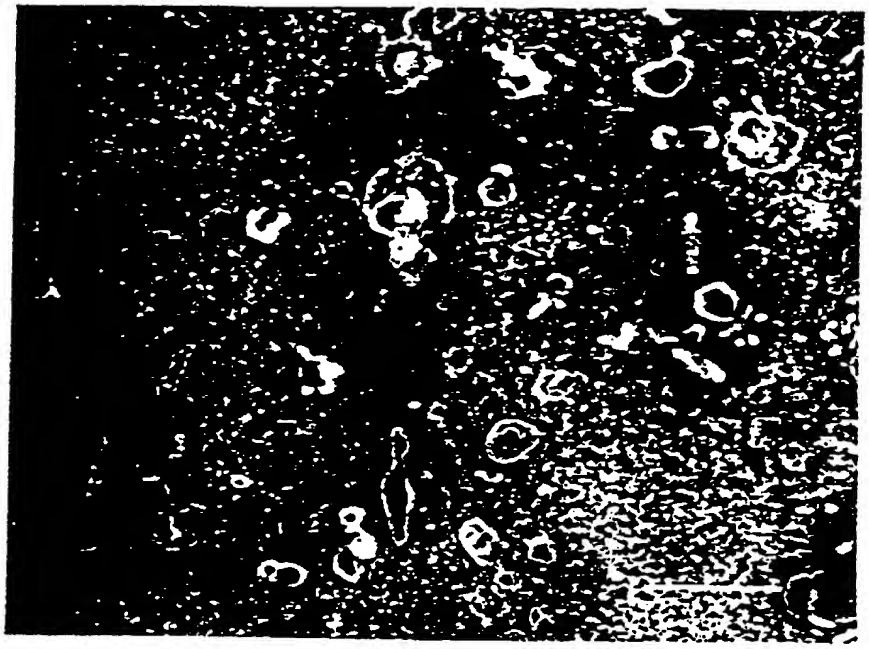

FIG.5

FIG.6

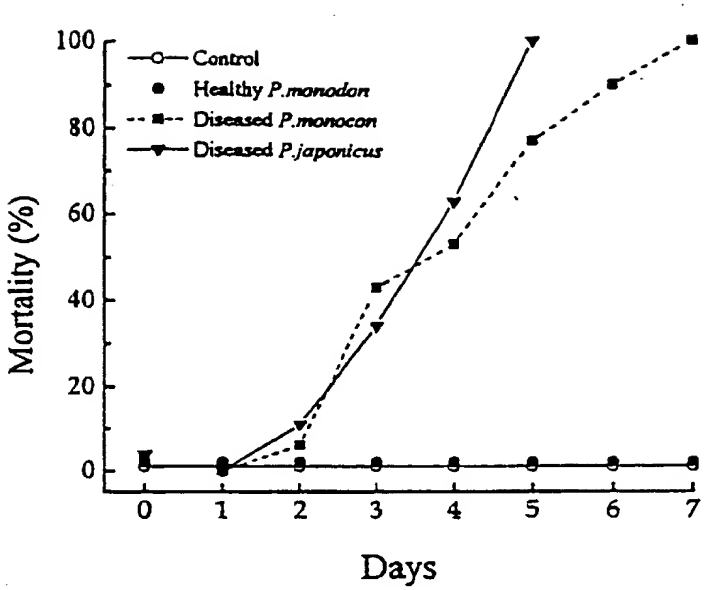

FIG.7

FIG. 8

FIG.9

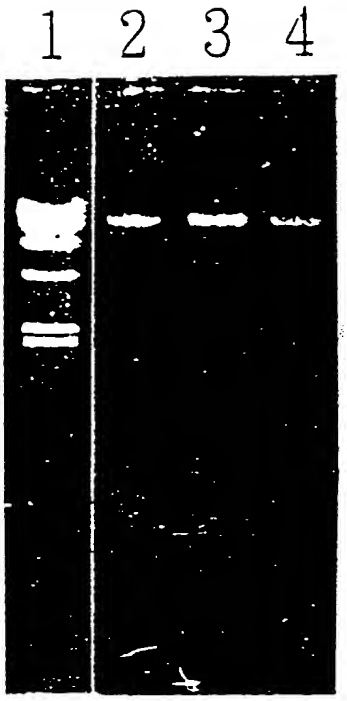

FIG.10

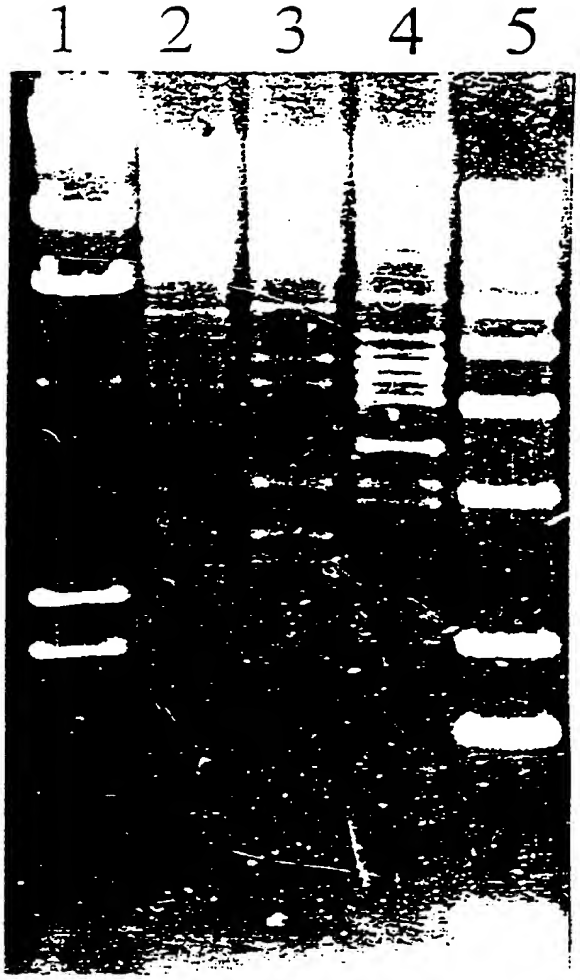

FIG.11

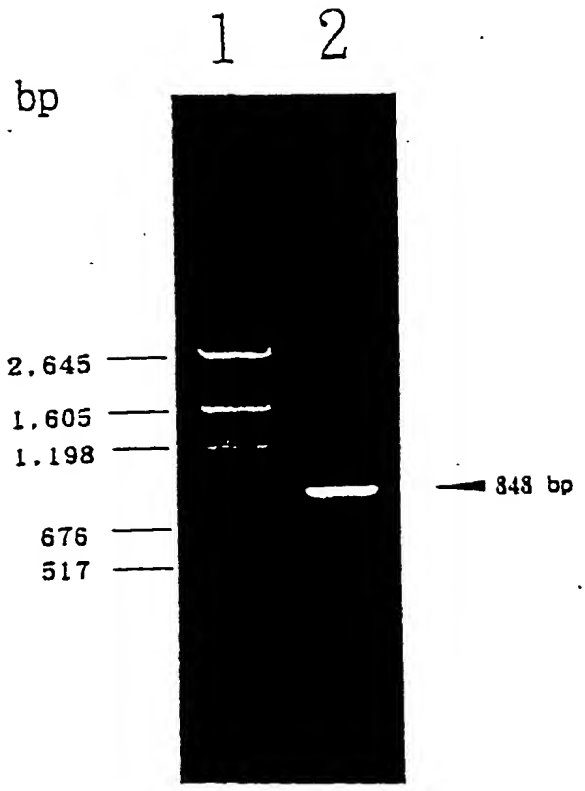

FIG.12

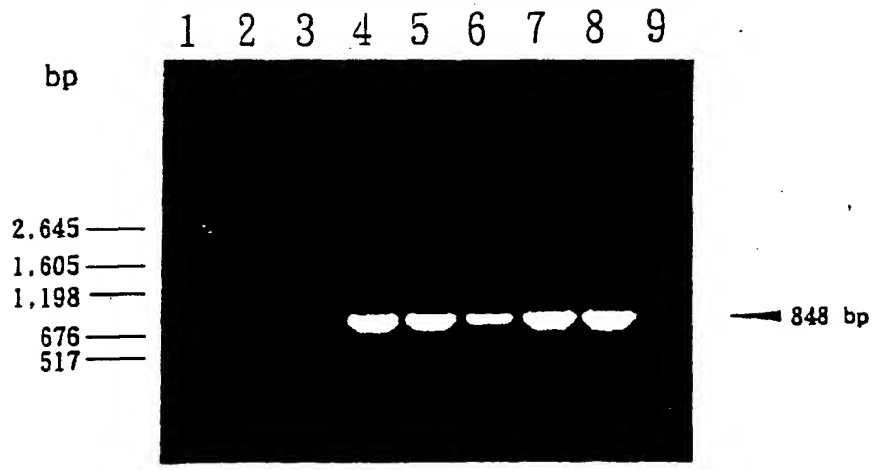

FIG.13

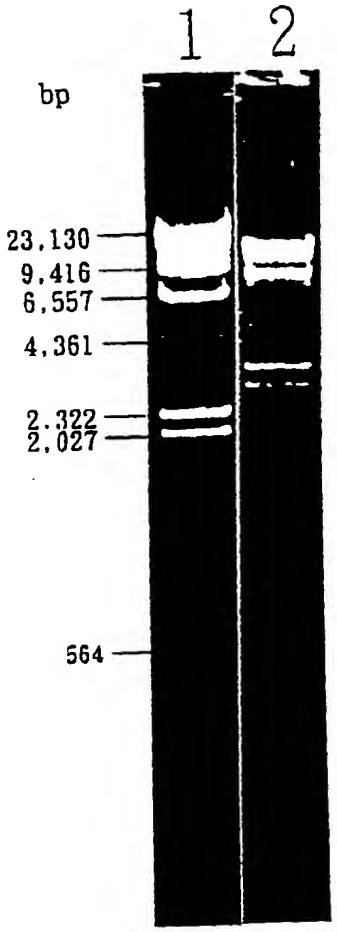

FIG.14

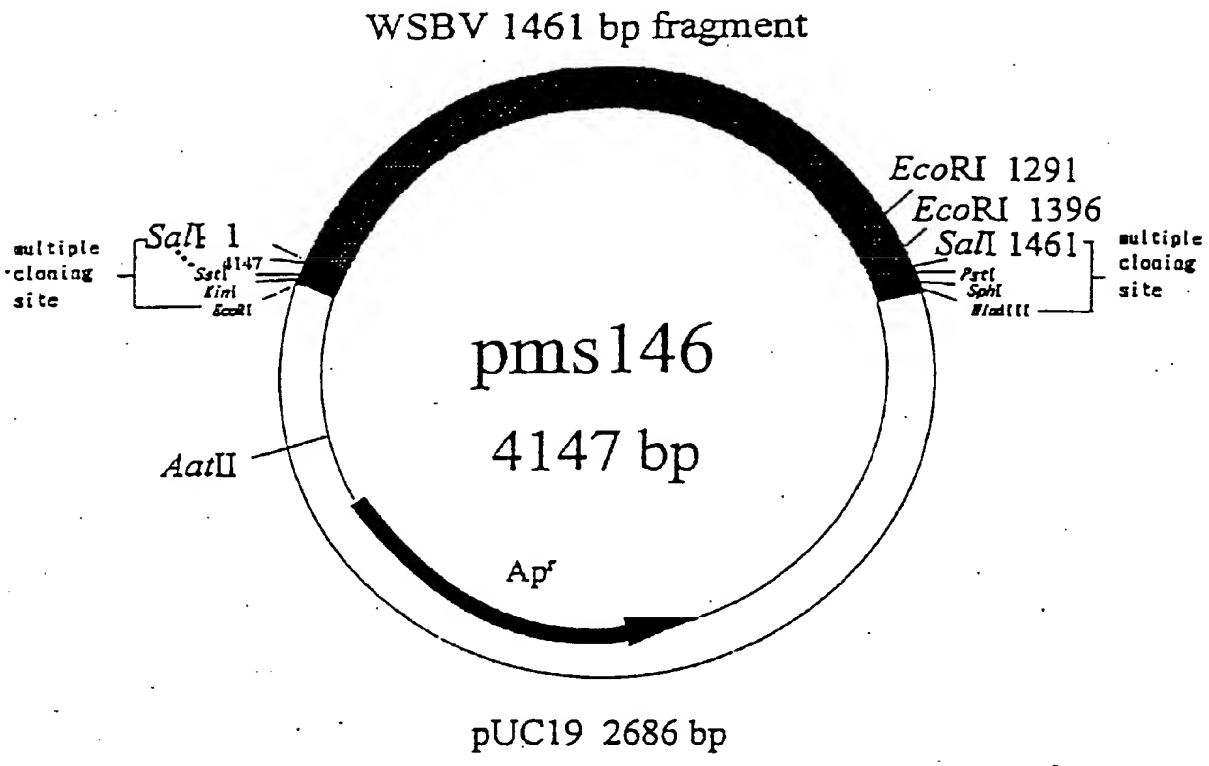

FIG.15A

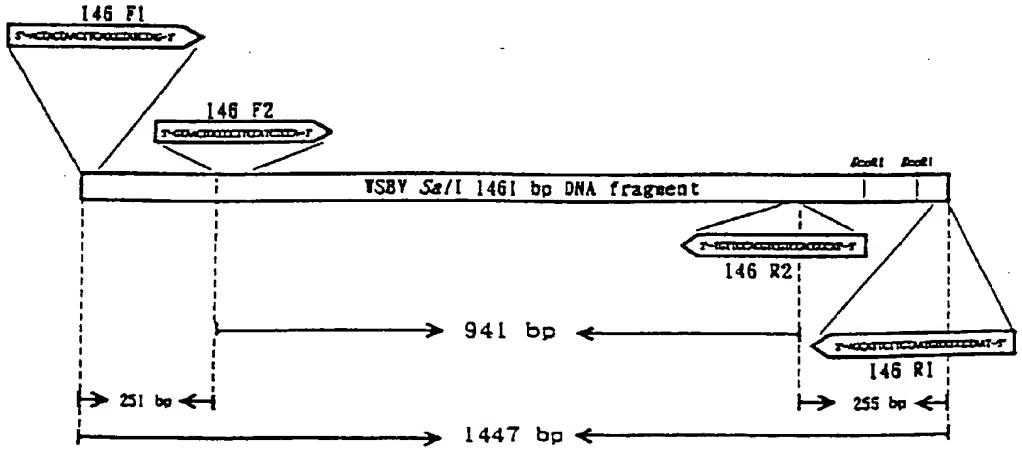

FIG.15B

```
1     GTCGA CAGAC TACTA ACTTC AGCCT ATCTA GTAAA ACAAG GTAAA AGATT
                      146 F1
51    CGACG GAGTT GACCC AGCCT TCGCT GCCGC CCTCA CCTGC GCTTC TCACC
101   TCATG CTTTC TTCCA TGGAT TCCCA TACAA AGTCA TCTTT CATGG ACAAC
151   ATCAA ATTGC ACATG ACTGA TACTC AATGC TTCTT CAAGA ACATT GAACG
201   ATTTG AGAAA TTCTT GGGAA GATAT GGGGA CGAAT ACGCC ATGTC CCACA
251   AGCAA AATTG TAACT GCCCC TTCCA TCTCC ACCAC ACTTT TACTC CCTCA
                          146 F2
301   GATAA CGAGC ATCTG GTATC CTCTT TCGCA TTCGC CCGCC CAGAA GTCTC
351   CATGG AAGAA ATTAG AGCCA CACCC TATCA GGCCA ACAAG CTTAT TAGTG
401   ACAAA CATTA CGTGA TGAAC ATGTC CAAGA TCGAT TCTAG AGTAA CAGGA
451   TCTTC CCTCC TTAAG AAGGT TAGCG AATGG ACTGA AATGA GAATG AACTC
501   CAACT TTAAT GGAAC ATTTG AACCA TCAAG ACTCG CCCTC TCCAA CTCTG
551   GCATG ACAAC GGCAG GAGTC AACCT CGACG TTATT GTCAA ACCAA ATAAT
601   GCAAG AAGTG TACTA GGAAT ATTGG AATGT CATCG CCAGC ACGTG TGCAC
651   CGCCG ACGCC AAGGG AACTG TCGCT TCAGC CATGC CAGCC GTCTT CCAGG
701   CAACC GATGG AAACG GTAAC GAATC TGAAC TGATC CAGAA TGCTC TGCCA
751   AGGAA CAGAT ACATC CAAAA GAGCA CAATG AACGC TCAAA CTGTC GTGTT
801   TGCTA ATGTT TTGGA ACAAC TTATC GCCGA TCTTG GAAAG GTTAT CGTGA
851   ACGAA CTGGC CGGCA CCATC GCTGA ATCTG TACCA GAAAG CGTAT ATGAA
901   AACAC CAAGG AAATG ATTGA TAGAC TAGGC TCTGA CGACC TCTTC AAATC
951   TAATA ATAAT GGAGG AGTAG AATCA ATGGA TTATG AAGAT AGCGA AACAA
1001  CATCC AACAA TGGTC CCGTC CTCAT CTCAG AAGCC ATGAA GAATG CCGTC
1051  TATCA CACAC TAATT TCCGG CAAGG CAGCT CGCCC GGAAA ATGTA CCATT
1101  CGCCT CATGC GCCAG CGGCC CTCTC GCCTT TGATT TCCTT CTGTC AAAGG
1151  GAGAT ACATT CGAAG AAAAG AACGC CGAAC AAGGT GCAGC AGCTG CCGTA
                          (146 R2)   tg ttcca cgtcg tcgac ggcat
1201  TCCTC TACCT ATTCT TCCTC TTCTA ACACT ACTCT TCGTA AGCAT TTGGC
                                                  EcoR1
1251  TCGAG TTTTC GAAGC CATCT CTAAG CAAGT AACTG ATGCT GAATT CAAGG
1301  ATATC CTCAA CGATA TCGAA CGTAA TATTT CTTCT GACTA TACTA ACTGT
                                                  EcoR1
1351  CCACC AAATA CTAAC CAAAA TGCCT TTGCT CTAGC TATCA AGAGA GAATT
1401  CAGCA GAATT GTTTC CTTCT TAACC ATTCT TCGTA AGAAC ATTAC ACCCG
                          (146 R1) 3'- agcat tcttg taatg tgggc
1451  CATTA GTCGA C
      gtaat-5'
```

FIG.15C

pms 146 primer set

pms 146 F1 5′ - ACT ACT AAC TTC AGC CTA TCT AG -3′

pms 146 R1 5′ - TAA TGC GGG TGT AAT GTT CTT ACG A -3′

pms 146 F2 5′ - GTA ACT GCC CCT TCC ATC TCC A -3′

pms 146 R2 5′ - TAC GGC AGC TGC TGC ACC TTG T -3′

pms 146 F3 5′ - TGG GAA GAT ATG GGG ACG AAT -3′

pms 146 R3 5′ - CGA AGA GTA GTG TTA GAA GAG GA -3′

pms 146 F4 5′ - AGA AGG TTA GCG AAT GGA CTG -3′

pms 146 R4 5′ - TTG AAG AGG TCG TCA GAG CCT -3′

pms 146 F5 5′ - GAA ACG GTA ACG AAT CTG AAC TG -3′

pms 146 R5 5′ - CAG TCC ATT CGC TAA CCT -3′

pms 146 R6 5′ - CGT CCC CAT ATC TTC CCA -3′

FIG.15D

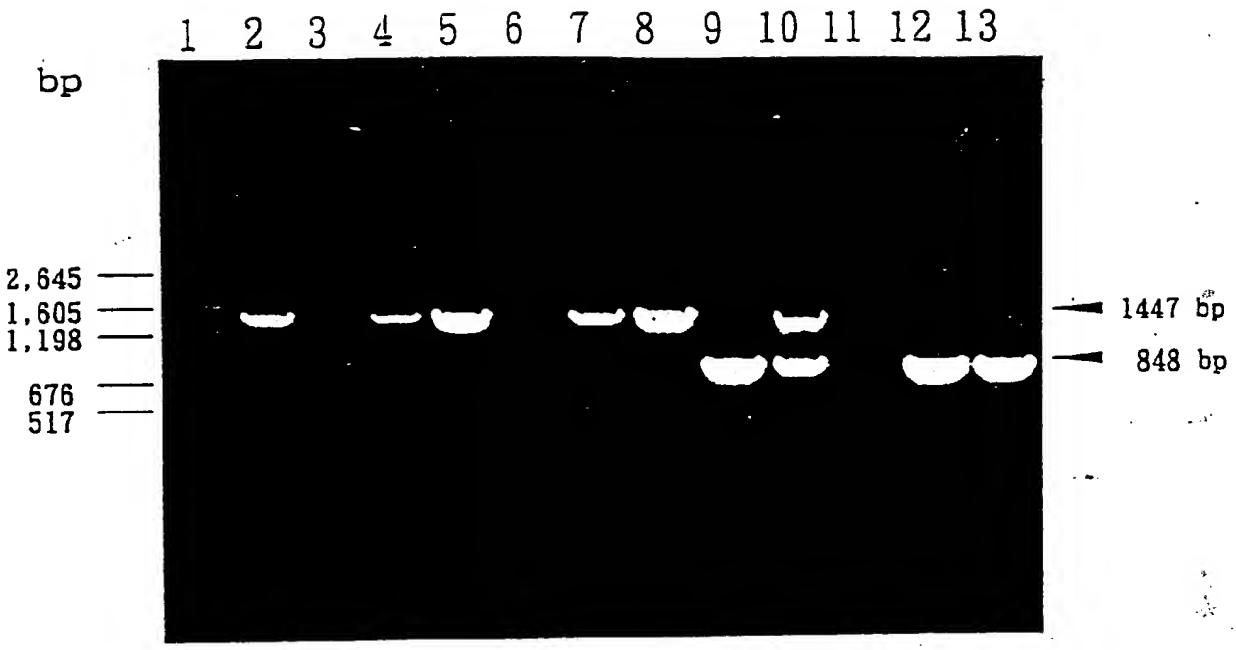

FIG.16

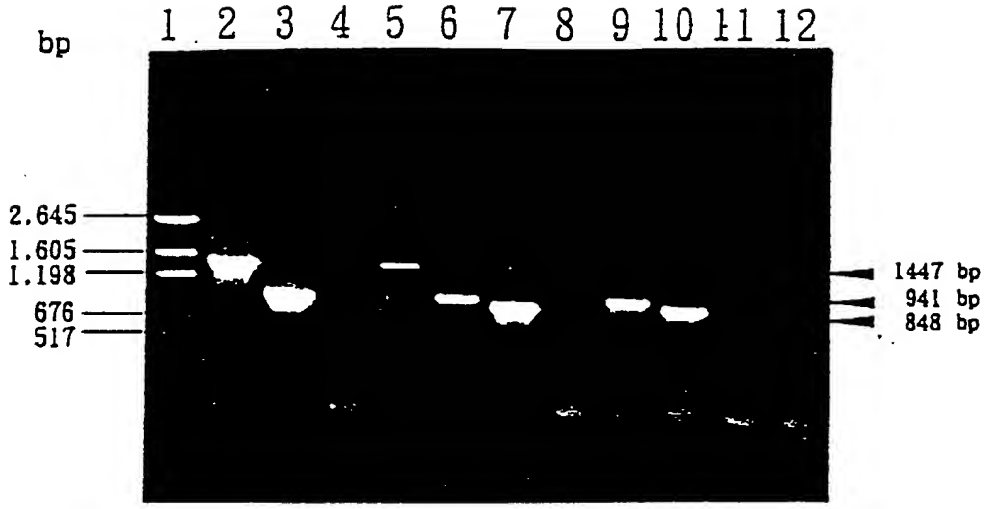

FIG.17

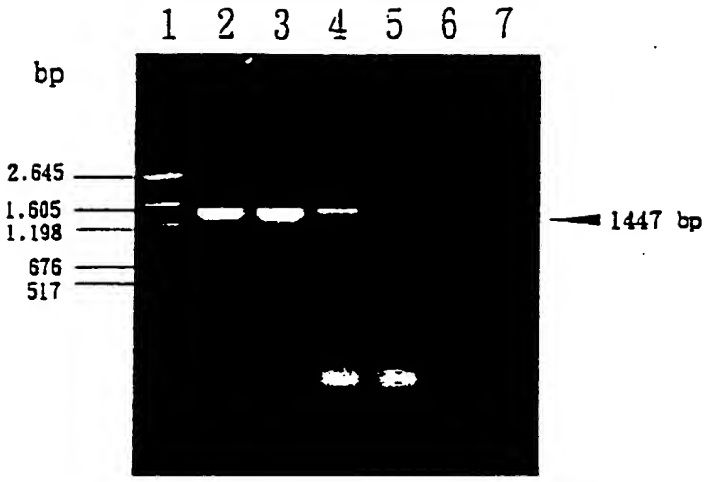

FIG.18

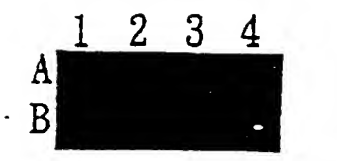

FIG.19

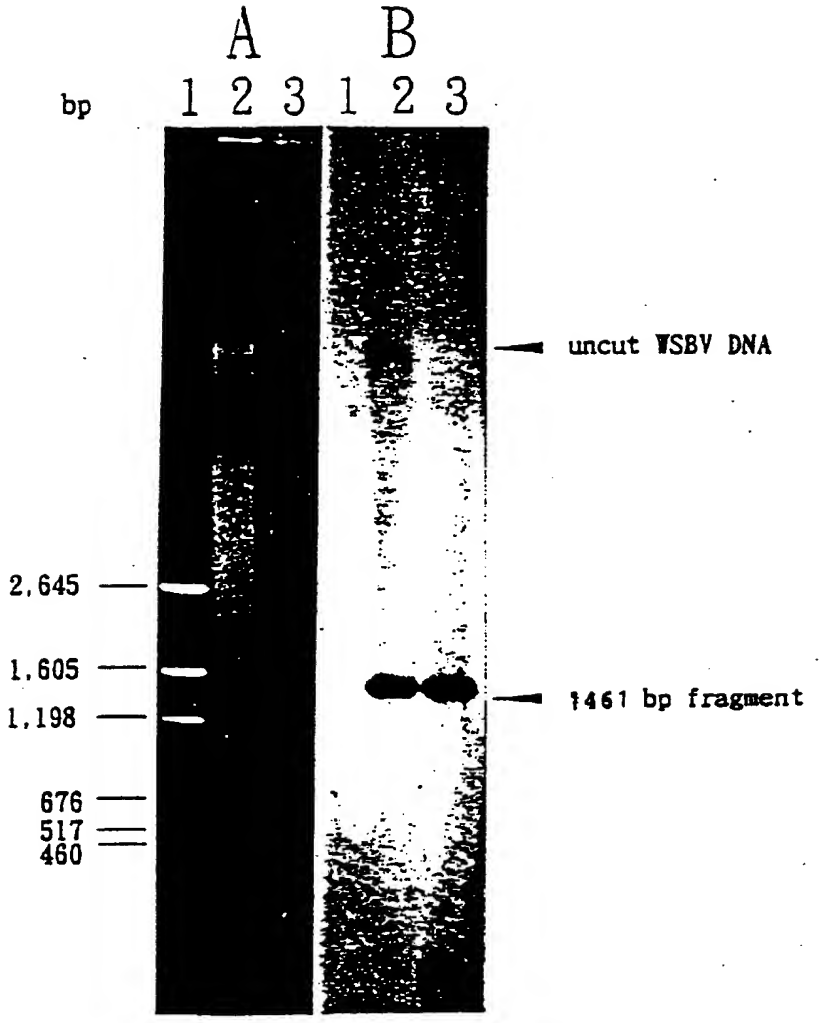

FIG.20

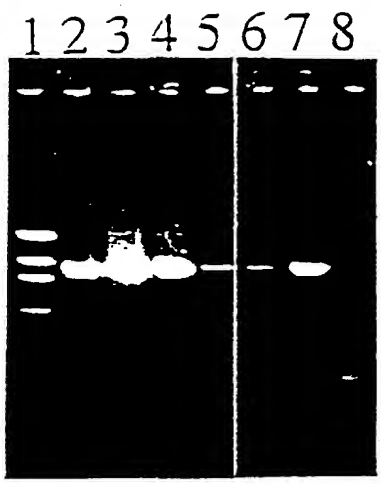

FIG.21

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 785 255 A3

(12) EUROPEAN PATENT APPLICATION

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14.10.1998 Bulletin 1998/42

(43) Date of publication A2:
23.07.1997 Bulletin 1997/30

(21) Application number: **97300242.1**

(22) Date of filing: **16.01.1997**

(51) Int Cl.[6]: **C12N 15/34**, C12N 7/00, C12N 7/02, C12Q 1/68, C12Q 1/70

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AT BE CH DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE

(30) Priority: **17.01.1996 US 587670**

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  Taipei (TW)
- **Lu, Chu-Fang**
  Taipei (TW)

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London WC1X 8AL (GB)

(54) Identification, purification and detection of WSBV (baculovirus associated with white spot syndrome)

(57) This invention relates to the identification, purification and detection of a new infectious viral agent in arthropods, especially shrimps. The virus is named as WSBV (Baculovirus associated with white spot syndrome). Two WSBV genomic DNA libraries were constructed and based upon the sequence of one of the cloned WSBV DNA fragments, a WSBV specific primer set for PCR to detect the WSBV infection in penaeid shrimps has been developed. The results of the present invention provide an effective diagnostic tool for screening WSBV infection in animal host organisms, in particular shrimps, to prevent the further spread of this viral disease.

EP 0 785 255 A3

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European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 97 30 0242

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A	WANG C-H ET AL: "Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of Penaeus monodon." DISEASES OF AQUATIC ORGANISMS 23 (3). 1995. 239-242, XP002074931 * the whole document * ---	1-27	C12N15/34 C12N7/00 C12N7/02 C12Q1/68 C12Q1/70
P,A	LO C-F ET AL: "Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction." DISEASES OF AQUATIC ORGANISMS 25 (1-2). 1996. 133-141, XP002074932 * the whole document * ---	1-27	
A	EP 0 596 508 A (BOYCE THOMPSON INSTITUE FOR PLANT RESEARCH) 11 May 1994 * the whole document * -----	1-27	
			TECHNICAL FIELDS SEARCHED (Int.Cl.6)
			C07K C12N C12Q

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	20 August 1998	Moreau, J

CATEGORY OF CITED DOCUMENTS

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NOTE

A comparative study of three different isolates of white spot virus

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ABSTRACT: Three separate isolates of white spot virus (WSV) purified from 3 different penaeid shrimp species from different countries were compared morphologically, biochemically, and genomically using the following techniques: negative stain electron microscopy, sodium dodecyl-sulfate polyacrylamide gel electrophoresis / western blot, and restriction fragment length polymorphism (RFLP), respectively. Under the electron microscope, the 3 isolates were indistinguishable. Their nucleoprotein cores exhibited the unique striated structure characteristic of the baculovirus-like agents associated with white spot syndrome. The dimensions of the nucleoprotein cores were also identical for all 3 isolates. SDS-PAGE gels of purified virus preparations showed all 3 to be identical in the position of at least 3 of the most prominent protein bands of WSV, with approximate molecular weights of 19, 23.5, and 27.5 kDa. Western blot analyses also revealed these 3 same protein bands in identical positions for all 3 isolates. RFLP analyses of the viral genomes using *Hind* III and *Eco*R I enzymes revealed that although the 3 isolates were identical when cut with *Eco*R I, the isolate from *Penaeus japonicus* from China was distinguishable from the other 2 genomes (*P. monodon* from Indonesia and *P. setiferus* from the U.S.) when cut with *Hind* III.

KEY WORDS: White spot virus · Penaeid shrimp

The white spot syndrome affecting penaeid shrimps has been responsible for huge losses in shrimp aquaculture in many shrimp growing countries of the world. Consequently, researchers from some of these countries (Japan, Thailand, Taiwan) have isolated and partially characterized baculovirus-like agents which appear to be variants of the same virus if not completely identical (Nadala et al. 1998). These non-occluded baculovirus-like agents have been previously called various names by the different laboratories that isolated them. The *Penaeus monodon* isolate in Thailand was called PmNOBII (*Penaeus monodon* non-occluded baculovirus) (Wongteerasupaya et al. 1995), the isolate from *P. monodon*, *P. japonicus* and *P. penicillatus* in Taiwan was called WSBV (white spot syndrome associated baculovirus) (Lo et al. 1996), the isolate from *P. japonicus* in Japan was called PRDV (penaeid rod-shaped DNA virus) (Inouye et al. 1996), and the isolate from *P. japonicus* in China was called CBV (Chinese baculovirus) (Lu et al. 1997, Nadala et al. 1998a). To minimize confusion, we will refer to all of these isolates as white spot virus (WSV). This nam recognizes the association of the virus with the white spot syndrome and at the same time avoids the issue of whether it is a baculovirus or not, which is at present still unresolved (Murphy et al. 1995).

Our laboratory had previously characterized an isolate of the white spot virus from *Penaeus japonicus* obtained from China (Lu et al. 1997). Subsequently, isolates were obtained from *P. monodon* from Indonesia and *P. setiferus* from the U.S. (Loh et al. 1998). In order to examine the relationships between these new isolates, and to determine whether or not they can be distinguished from each other, a comparative study was made of their morphology, structural viral proteins and viral genomes.

Materials and methods. The virus isolates came from gill and head soft tissues of infected farm-raised 5 to 10 g *Penaeus japonicus* from China, farm-raised adult 80 to 100 g (head only) *P. monodon* from Indonesia, and wild-caught 5 to 10 g *P. setiferus* from South Carolina, USA. The Chinese and U.S. viral isolates were propagated in white shrimp (50 to 60 g *P. vannamei*) before virus purification. Head soft tissue filtrates [200 µl of 4% w/v in TNE (0.05M Tris, 0.1 M NaCl, 1 mM EDTA) prepared from WSV-infected shrimp were inoculated by intramuscular injection into the second abdominal segment of the shrimps. Two to four days after injection, moribund specimens were collected and stored at –80°C.

To purify the virus, 80 g of gill and/or head soft tissues were harvested from frozen infected shrimps. The tissues were suspended in TNE at 10% w/v and

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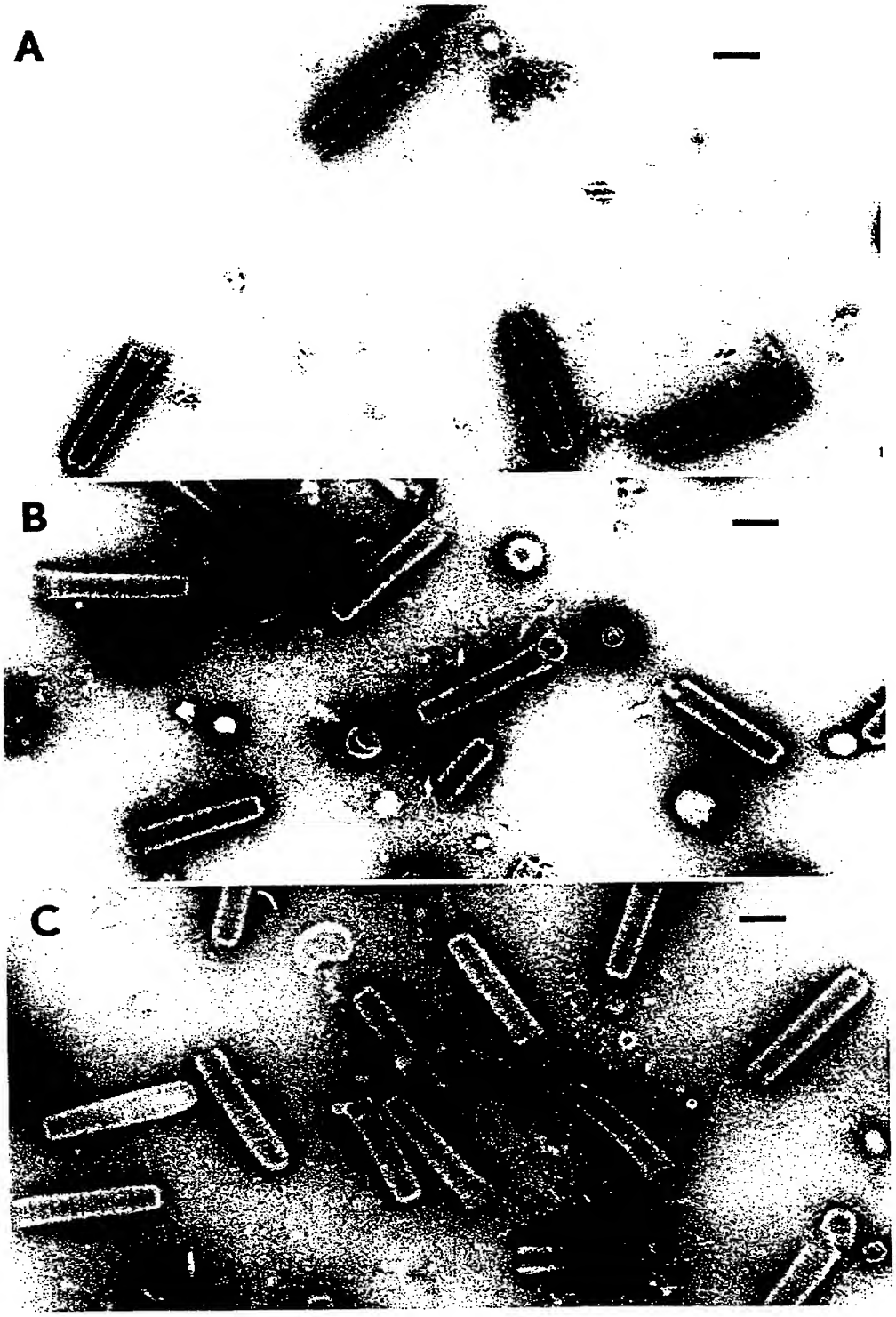

Fig. 1. Electron micrographs of negatively stained WSV particles. (A) Indonesian isolate. (B) U.S. isolate. (C) Chinese isolate. All scale bars = 100 nm

homogenized [Brinkmann Polytron 3000 (Kinematica AG) with 90/Polytron PT-DA 3012/2TS] (Brinkmann Instruments Inc., NY, USA). Tissue debris was pelleted twice at 4946 × *g* for 10 min. Virus was pelleted from the supernate at 73 360 × *g* for 20 min, resuspended in 12:1 volume of TNE, and clarified at 2000 × *g* for 20 min. Virus was again pelleted from the supernate at 73 360 × *g* for 20 min, resuspended in 2 ml TNE, layered on top of a 30–40–50% (w/v) CsCl gradient and centrifuged at 192 000 × *g* for 19 h at 4°C. The bands of virus located in the lower half of the tube were collected, diluted 1:6 in TNE, checked for purity by electron microscopy (EM), and pelleted at 73 360 × *g* for 30 min. The pellet was resuspended in 200 µl PBS and stored at −80°C until needed. For EM examination, virus samples were mounted on formvar-coated, carbon-stabilized copper grids (200 mesh), negatively stained with 2% (w/v) uranyl acetate, and examined under a Zeiss EM10/A electron microscope.

The structural proteins of the different virus isolates were analyzed by 12.5% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) according to the method of Laemmli (1970). Samples were electrophoresed for 40 min at 200 V and the gels stained using the silver stain plus kit (Biorad, CA, USA). Western blots were performed according to previously published protocols (Nadala et al. 1997).

The WSV DNA was extracted from purified virus using the QIAamp Tissue Kit (QIAGEN Inc., CA, USA). Aliquots of the DNA (100 to 300 ng in 17 µl) were digested with excess *Hind* III (10 units) or *Eco*R I (12 units) overnight at 37°C. Digested DNA was then subjected to electrophoresis in an agarose gel (0.7% w/v in TBE buffer) at 30 V for 4 to 7 h.

Results and discussion. Electron microscopical examination of negatively stained CsCl-banded virus particles revealed the same conspicuous vertical striations in all 3 different isolates of WSV (Fig. 1). The dimensions of the nucleoprotein core structures were also virtually identical at 316 to 350 nm in length and 65 to 70 nm in diameter. Analysis of the structural proteins of the 3 virus isolates by SDS-PAGE gel electrophoresis of purified virus particles revealed that at least 3 of the prominent and consistent proteins of WSV (19, 23.5, and 27.5 kDa) were in identical positions in the gel (Fig. 2A, arrowheads). Western blot analysis of the 3 virus isolates also showed these same 3 proteins to be in the same positions and with comparable staining (Fig. 2B, arrowheads). These results show that the 3 different isolates of WSV were virtually identical morphologically, by protein profile, and even antigenically by western blot.

When the purified DNA of the 3 isolates were digested with *Eco*R I and electrophoresed, 36 bands were distinguished. All 3 isolates showed the same pattern of bands (Fig. 3). On the other hand, when the purified DNA of each of the 3 isolates was digested with *Hind* III and electrophoresed, 31 bands were distinguished. When compared to each other, the isolate from China had an additional band at 15.5 kb (Fig. 3, arrowhead) which was missing in the other 2 isolates. The isolates from Indonesia and the U.S. had an additional band at 10 kb (Fig. 3, arrowhead) which was missing in the Chinese isolate. This could mean that the Indonesian and U.S. isolates are more related variants than the Chinese isolate. Our laboratory is presently doing more extensive RFLP analyses utilizing several enzymes and double digests as well as

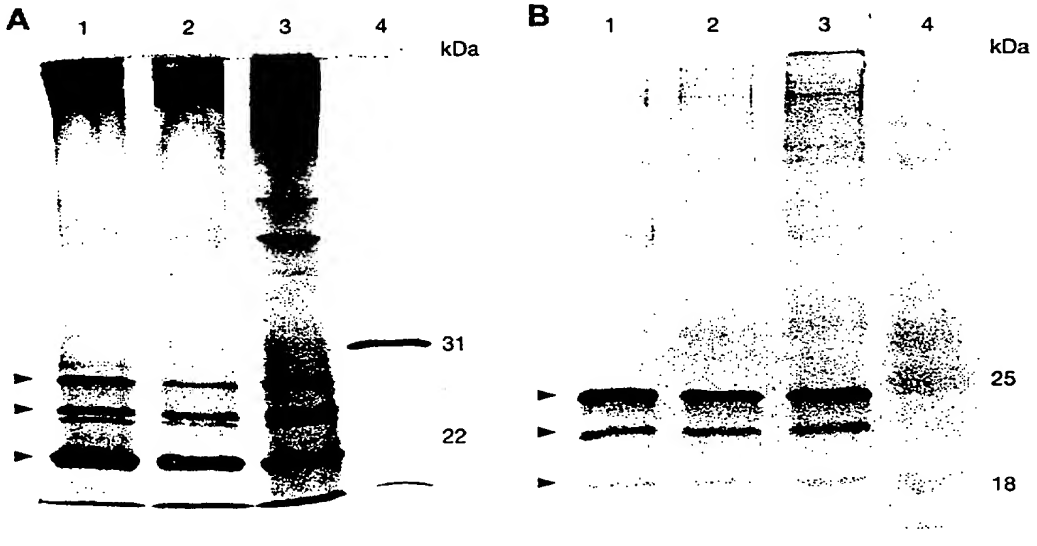

Fig. 2. SDS-PAGE and western blot of purified WSV isolates. (A) Silver-stained gel. Lane 1: U.S.; Lane 2: Indonesia; Lane 3: China; Lane 4: Biorad low molecular weight markers. (B) Western blot. Lane 1: U.S.; Lane 2: Indonesia; Lane 3: China; Lane 4: Biorad prestained broad range markers

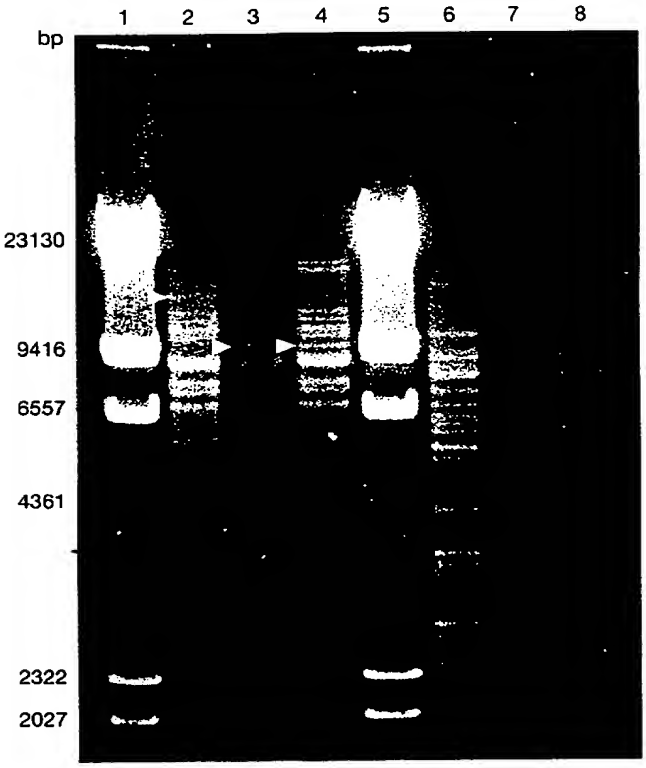

Fig. 3. Agarose gel electrophoresis of *Hind* III and *Eco*R I digested DNA from WSV isolates. Lanes 1 & 5: *Hind* III digested Lambda DNA markers; Lane 2: China, *Hind* III digest; Lane 3: Indonesia, *Hind* III digest; Lane 4: U.S., *Hind* III digest; Lane 6: China, *Eco*R I digest; Lane 7: Indonesia, *Eco*R I digest; Lane 8: U.S., *Eco*R I digest

nucleic acid sequencing to obtain a more meaningful comparison of the different isolates of WSV.

The *Hind* III restriction digest pattern of the WSV isolate of Wang et al. (1995) show that it is more related to the Chinese isolate than to the other 2 because of the presence of the 15.5 kb band, which they estimate at 16.9 kb in size. Despite the slight discrepancy in our size estimates, the general pattern seems to be identical with the Chinese isolate. It is not possible to compare at this time the restriction enzyme patterns of the isolates described by Wongteerasupaya et al. (1995) and Inouye et al. (1996) because *Hind* III digests of their isolates are not yet available.

Based on the total size of the restriction fragments, we estimate the size of the WSV genome at around 190 to 200 kb. This figure is slightly bigger than our previously published estimate (183.9 kb) (Nadala et al. 1998) because 2 fragments (11.75 and 2.4 kb) which migrated close to similarly sized fragments were missed in our earlier count.

The results of this study indicate that WSV can infect many different penaeid species in different areas of the world. It also supports the theory that WSV is the non-occluded baculovirus-like agent associated with white spot syndrome that has been isolated by different laboratories throughout the world. These different isolates are probably variants of WSV distinguishable only by their nucleic acid sequence. The finding of WSV in *Penaeus setiferus* is also worrisome as this particular penaeid species is becoming increasingly utilized in shrimp aquaculture.

Acknowledgements. This study was supported by grants from the University of Hawaii Sea Grant and College Program, Institutional Grant No. NA36RG0507, UNIHI-SEAGRANT-JC-98-04, and the Aquaculture Development Program, Department of Land and Natural Resources, State of Hawaii Contract No. 38066.

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Specific genomic DNA fragment analysis of different geographical clinical samples of shrimp white spot syndrome virus

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ABSTRACT: White spot syndrome (WSS) has been found in many species of shrimp and crabs, not just in Asia but globally. The causative agent is known as white spot syndrome virus (WSSV). In order to clarify the relatedness of WSSV from various geographic regions, we compared the viral DNA of a number of clinical samples of WSSV: (1) China96-116A from *Penaeus chinensis*, (2) India95-314 from *Penaeus monodon*, (3) grocery store95-204 and grocery store96-115 from *P. monodon* possibly originating from Thailand, (4) crayfish97-25 from *Orconectes punctimanus* collected from the U.S. National Zoo, (5) Thailand95-46 from experimentally infected *Penaeus vannamei*, (6) South Carolina97-64 from *P. vannamei*, and (7) Texas95-242 and Texas96-7 from *P. vannamei*. These specimens were first examined by dot hybridization analysis with nucleic acid probes derived from a WSSV Taiwan isolate. Although the intensity of the hybridization signals varied, and although some specimens of India95-314, crayfish97-25, Texas95-242 and Texas96-7 failed to give a detectable hybridization signal with certain probes, the broad consistency of dot hybridization data suggests that these WSSV clinical samples from different geographical locations are closely related. Following this analysis, all the specimens were examined using 10 virus-specific polymerase chain reactions (PCR). The amplification products were subsequently digested with *Cfo* I, *Hae* III, *Hpa* II and *Rsa* I restriction endonucleases to determine if there were any DNA fragment polymorphisms in the WSSV clinical samples. The results highlighted the genetic relatedness of all the WSSV clinical samples with the possible exception of a series of Texas viral samples which could be distinguished from the other geographic samples in some of the PCR-based tests.

KEY WORDS: Genomic DNA analysis · Restriction profiles of specific viral DNA fragments · White spot syndrome virus · WSSV · WSBV · Geographical clinical samples

INTRODUCTION

The commercial cultivation of marine shrimp is now a global industry, and one of the biggest problems facing this industry is disease. White spot syndrome (WSS) is a viral disease which affects many commercially cultivated marine shrimp species, not just in Asia but globally (Lightner 1996, Flegel 1997). The principal clinical sign of WSS is the presence of white spots on the proximal surface of the cuticle of the diseased shrimp. Affected individuals become lethargic and go off their feed. In shrimp ponds, they congregate in the shallows along the edges of the pond, and in culture tanks they sink inactively to the bottom, where they are frequently attacked and cannibalized by the healthier shrimp. WSS can cause up to 100% mortality (Chou et al. 1995, 1998), which leads to a correspondingly devastating economic impact. WSS has been formally recognized since 1992, but so far no significant resistance to this disease has been reported for any of the penaeid species (Lightner 1996).

The causative agent of WSS has, however, been identified: it is a non-occluded rod-shaped virus known

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as white spot syndrome virus (WSSV) or white spot 'baculovirus' (WSBV) (Wang et al. 1995, Wongteerasupaya et al. 1995, Lightner 1996, Lo et al. 1997). Although WSS has been observed in many species of shrimps and crabs from various geographic areas, for some time it has remained uncertain whether the causative agents of WSS are really identical or at least closely related, or whether, in fact, there are not several geographic strains or even several distinct viruses. Previous analysis has argued for a close relatedness for clinical samples of WSSV from different species of shrimps and crabs (Lo et al. 1996a,b), but all the tested specimens in these studies were collected from shrimp farms or coastal waters in the same geographic area of Taiwan. In the present study, therefore, to clarify the relatedness of different geographical clinical samples of WSSV, the viral DNA of a number of clinical samples of presumptive WSSV collected from Taiwan, China, India, Thailand, and the USA were compared using dot hybridization analysis with 11 distinct nucleic acid probes (3 to 12 kbp) derived from the inserts of plasmid clones selected from *Sal* I and *Hind* III genomic libraries of a Taiwan WSSV isolate from *Penaeus monodon* (Lo et al. 1996a).

Random amplification of polymorphic DNA (RAPD) is a widely used assay of DNA polymorphism in which RAPD profiles and/or markers distinguish between different geographic isolates and identify genetic variation within groups (J. G. Williams et al. 1990, 1992, Welsh & McClelland 1990, C. L. Williams et al. 1994). To date, however, neither markers nor an RAPD assay have been developed for WSSV. In fact, currently there are almost no RAPD assay systems that are suitable for use with unpurified virus because of the possibility that the host DNA might influence the reaction. On the other hand, without an adequate *in vitro* culture system for shrimp viruses, plaque purification is impossible, so it is difficult to get a pure culture of each virus isolate. In the present study, therefore, specific DNA fragments were used instead for a restriction profile analysis of the unpurified WSSV clinical samples. The primers used to amplify specific DNA fragments and to investigate the restriction fragment length polymorphism (RFLP) were 20 to 25 nucleotides in length and based on the sequences derived from Taiwan WSSV DNA fragments from 9 distinct plasmid clones. Such long and specific primers usually generate an anticipated PCR product only if the clinical sample is specific to the primer.

MATERIALS AND METHODS

Virus. The geographic clinical samples of WSSV, all of which were from Dr Lightner's collection, were as follows: (1) China96-116A from *Penaeus chinensis*, (2) India95-314 from *Penaeus monodon*, (3) grocery store95-204 and grocery store96-115 from *P. monodon* possibly originating from Thailand, (4) crayfish97-25 from *Orconectes punctimanus* collected from the U.S. National Zoo, (5) Thailand95-46 from experimentally infected *Penaeus vannamei*, (6) South Carolina97-64 from *P. vannamei*, and (7) Texas95-242 and Texas96-7 from *P. vannamei*.

As we only discovered after the first battery of tests (see 'Results': Table 2), the original grocery store95-204 samples (which were somewhat degraded in storage) unexpectedly failed to hybridize with any of the probes. A second set of specimens (grocery store96-115) was therefore tested separately. Conversely, the South Carolina specimens showed hybridization patterns which were surprisingly similar to the Asian clinical samples. Two other specimens from the same South Carolina lot were therefore also subjected to a subsequent batch of tests along with 2 other American clinical samples, Texas95-242 and Texas96-7.

A WSSV Taiwan clinical sample (Lo et al. 1996a) was used as a positive control while WSSV-free shrimp tissues were used as negative controls throughout the experiment. DNA extracted from *Baculovirus penaei*-infected (BP-infected) shrimp tissues was also included as a negative control in some of the dot hybridization analyses and WSSV diagnostic PCR (see 'Results': Table 2).

Extraction of viral DNA. A piece (approx. 100 to 200 mg) of tissue (gills, appendages, midgut, hepatopancreas or muscles) or an aliquot (100 µl) of hemolymph from shrimp collected from various geographic areas was placed directly in a microfuge tube containing 1.2 ml digestion buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8, 25 mM EDTA, pH 8, 0.5% N-lauryl sarcosine, 0.5 mg ml^{-1} proteinase K) and crushed with a disposable stick. After 1 h incubation at 65°C, 600 µl of the supernatant of the DNA/digestion buffer mixture was collected, and 100 µl of 5 M NaCl and 70 µl of CTAB/NaCl stock solution (10% N-cetyl N,N,N-trimethylammonium bromide in 0.7 M NaCl) were then added. Following incubation at 65°C for 10 min, the DNA was extracted with an equal volume of chloroform/isoamyl alcohol once, followed by an equal volume of phenol 2 to 3 times, and a double volume of chloroform/isoamyl alcohol once. The DNA was recovered by ethanol precipitation, dried and resuspended in 100 µl of 0.1 × TE buffer (1 × TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 7.6) at 65°C for 30 min, and then stored at 4°C until used for dot hybridization and PCR.

Detection of WSSV in collected shrimp samples by WSSV diagnostic PCR with the use of pms146F1/R1 primer set. A primer set derived from the sequence of a cloned WSSV *Sal* I 1461 bp DNA fragment (Lo et al.

1996a) consisting of pms146F1 (5'-ACT ACT AAC TTC AGC CTA TCT AG-3') and pms146R1 (5'-TAA TGC GGG TGT AAT GTT CTT ACG A-3') was utilized for 1-step WSSV diagnostic PCR (Lo et al. 1996a) in order to confirm the presence of the virus in the collected shrimp samples (pms: *Penaeus monodon* WSSV *Sal* I fragment). The 1-step PCR was performed as follows. The DNA samples used for amplification totaled 0.1 µg in a 100 µl reaction mixture containing 10 mM Tris-HCl, pH 8.8 at 25°C, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 µM of each dNTP, 100 pmol of each primer, and 2 units of *Taq* DNA Polymerase (Life Technologies). The amplification was performed in a thermal cycler (Perkin-Elmer Corporation) for 1 cycle of 94°C for 4 min, 55°C for 1 min, 72°C for 2 min, then 39 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, plus a final 5 min extension at 72°C after 40 cycles. Control reactions containing no template DNA were run for all PCR reactions. A portion (10 µl) from each of the completed PCR reactions was mixed with 1 µl loading buffer and subjected to electrophoresis on 1% agarose gels containing ethidium bromide at a concentration of 0.5 µg ml^{-1}, and visualized by ultraviolet transillumination.

As described previously (Lo et al. 1996a), the quality of the DNA extracted from the collected shrimp samples was checked with a primer set amplifying a decapod gene before the application of WSSV diagnostic PCR. For this purpose, 2 primers, 143F (5'- TGC CTT ATC AGC TNT CGA TTG TAG-3', where N represents G, A, T or C) and 145R (5'-TTC AGN TTT GCA ACC ATA CTT CCC-3'), derived from a highly conserved region of the 18S rRNA sequence of decapods (Kim & Abele 1990, Lo et al. 1996a) were used.

Dot blot hybridization analysis. DNA samples were boiled for 10 min and then quenched on ice. An aliquot (1 µl) of each of the DNA samples was dotted onto a sheet of positively charged nylon paper (Boehringer Mannheim, Mannheim, Germany) that had been presoaked with 5 × SSC (1 × SSC = 150 mM NaCl, 15 mM Sodium Citrate, pH 7.0) for 5 min and air dried. After cross-linking the DNA with the membrane by UV light, the blot was used for hybridization with 11 WSSV probes which were non-radioactively labeled with digoxigenin (DIG)-dUTP (Boehringer Mannheim) by a random priming method. Following prehybridization at 65°C for 1 h in prehybridization solution (10 ml 5 × SSC with 100 mg of blocking reagent II, 50 µl of 20% sarkosyl, 20 µl of 10% SDS), the blot was hybridized at 65°C for 16 h with DIG-labeled probes. The detection of the DIG-labeled nucleotides in blots was accomplished by an immunological method using anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) and CSPD®, (Bo hringer Mannheim) as a chemiluminescent substrate for alkaline phosphatase. The blot was exposed to Kodak X-OMAT film at room temperature for 1 to 10 min to record the chemiluminescent signal. In this study, the blots were reused several times for hybridization analysis with different probes. For reprobing, the blots were rinsed in water for 1 min, treated with an alkaline solution (0.2 N NaOH, 1% SDS) at 37°C for 30 min, and then rinsed in 2 × SSC. Following the prehybridization, the blot was then hybridized with a new probe.

The inserts of 11 plasmid clones were used as probes: pms473 (12 kbp), pms484 (8 kbp), pms100 (6 kbp), pms321 (4.4 kbp), pms146 (1.5kbp), pmh2 (>9 kbp), pmh10 (>9 kbp), pmh11 (>9 kbp), pmh13 (>9 kbp), pmh32 (>9 kbp), pmh34 (>9 kbp). All these clones were selected from *Sal* I and *Hind* III genomic libraries of a Taiwan WSSV clinical sample (Lo et al. 1996a). The figures in parentheses indicate the size of the insert of each clone. The first set of DNA samples (1 to 47, see Table 2), i.e. the Asian and the first 2 South Carolina specimens, were analyzed with all 11 probes. Only 3 probes were used, however, for the second batch of DNA samples (48 to 71, see Table 3), which included the new grocery store and South Carolina as well as the 2 Texas clinical samples.

Analysis of the DNA of WSSV geographical clinical samples by PCR with specific primer sets and restriction fragment length polymorphism. We used 10 primer pairs (Table 1) based on the DNA sequences (unpubl. data from Dr Guang-Hsiung Kou's laboratory) of 9 plasmid clones (pms54, pms94, pms98, pms120, pms146, pms321, pms473, pmh11, pmh13) selected from *Sal* I and *Hind* III genomic libraries of a Taiwan WSSV isolate from *Penaeus monodon* (Lo et al. 1996a). Ten amplicons, Apms54F1/R1 (0.96 kbp), Apms94F1/R1 (0.94 kbp), Apms98F1/R1 (0.95 kbp), Apms120F1/R1 (0.95 kbp), Apms146F1/R1 (1.5 kbp), Apms321F1/R1 (0.9 kbp), Apms321F2/R2 (1.0 kbp), Apms473F3/R3 (1.1 kbp), Apmh11F1/R1 (1.0 kbp) and Apmh13F1/R1 (1.2 kbp), were expected with 1-step PCR and the primer sets. The size of each anticipated PCR product is indicated in parentheses. We used these 10 primer pairs and PCR to analyze the 71 DNA samples of various geographic WSSV clinical samples. To confirm that the amplified fragments were indeed virus specific, an additional internal primer set, pms98F2/R2 (Table 1), was also used to perform 2-step PCR. For Texas specimens, we also used Southern hybridization with probes prepared from PCR products of Taiwan WSSV isolate using internal primer sets of pms98 and pms146 to detect WSSV-specific PCR products using a method described previously (Lo et al. 1996a,b). The *Cfo* I, *Hae* III, *Hpa* II, and *Rsa* I restriction profiles of some specific viral DNA fragments were also compared.

The thermal cycling program and reaction conditions for 1-step PCR were the same as those described

Table 1. PCR primer sequences used in this study

Primer set	Primer sequence
1. 143/145	143F: 5'-TGCCTTATCAGCTNTCGATTGTAG-3' 145R: 5'-TTCAGNTTTGCAACCATACTTCCC-3'[a]
2. pms54F1/R1	pms54F1: 5'-CGTAACAGGCTCGGTGCC-3' pms54R1:5'-CAGCACGGATACGTTAAC-3'
3. pms94F1/R1	pms94F1: 5'-CGGTCTCAGTAATTCGTC-3' pms94R1: 5'-CCTCCATTTGCTGCAGTG-3'
4. pms98F1/R1	pms98F1: 5'-GACAATGTTGGTATCGGTAG-3' pms98R1: 5'-GAGCACGAGAAGCACGAC-3'
5. pms120F1/R1	pms120F1: 5'-GACATATACGCCACCAAGG-3' pms120R1: 5'-GGCAGCGTCCATACTGTTC-3'
6. pms146F1/R1	pmsI46F1: 5'-ACTACTAACTTCAGCCTATCTAG-3' pms146R1: 5'-TAATGCGGGTGTAATGTTCTTACGA-3'
7. pms321-1F1/R1	pms321F1: 5'-CGCCACCAAGGAATTCGAAC-3' pms321R1: 5'-GCAGACATGGCAGCGTCC-3'
8. pms321-2F1/R1	pms321F2: 5'-GCGAGCGGCGTACTACGAC-3' pms321R2: 5'-GAGGCCACAGCCGAAGCTG-3'
9. pms473F1/R1	pms473F3: 5'-AAGAGGAGGATTCTCCAGATCC-3' pms473R3: 5'-CCAACACGGTACACGTAATTC-3'
10. pmh11F1/R1	pmh11F1: 5'-GGTGATTCTGCATCCAGC-3' pmh11R1: 5'-GCGGATTCTATGAGGCGAG-3'
11. pmh13F1/R1	pmh13F1: 5'-CAGGATGGTACAGAGGAC-3' pmh13R1: 5'-GTCAATATAGCCATGGATGG-3'
12. pms98F2/R2	pms98F2: 5'-CTGGGCCGTAAAGTAGTG-3' pms98R2: 5'-CTGGACAATGCATGATGAG-3'

[a]N represents G, A, T or C

above for WSSV diagnostic PCR using the pms146F1/R1 primer set. For 1-step PCR, 10 µl of the 1-step PCR reaction mixture was added to 90 µl of PCR cocktail containing the inner primer pair, and this was then subjected to a second step of amplification in a thermal cycler (Perkin-Elmer Corporation) for 1 cycle of 94°C for 4 min, 55°C for 1 min, 72°C for 2 min, then 39 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, plus a final 5 min extension at 72°C after 40 cycles. Control reactions containing no template DNA were run for all PCR reactions. A portion (10 µl) from each of the completed PCR reactions was mixed with 1 µl loading buffer and subjected to electrophoresis on 1% agarose gels containing ethidium bromide at a concentration of 0.5 µg ml^{-1}, and visualized by ultraviolet transillumination.

RESULTS

Detection of WSSV in collected shrimp samples by WSSV diagnostic PCR with the use of pms146F1/R1 primer set

All the prepared templates were amplifiable when assessed by the shrimp DNA-specific primer set 143/145 (Tables 2 & 3). Of the 47 tested DNA samples shown in Table 2, only the 3 grocery store95-204 specimens consistently gave negative results in 1-step WSSV diagnostic PCR. As shown in Table 3, however, 10 of the Texas specimens gave negative results, and only 1 Texas specimen was positive in 1-step WSSV diagnostic PCR.

Dot blot hybridization analysis

In Table 2, apart from grocery store95-204, only India95-314#3 and crayfish97-25#1 failed to hybridize with all 11 WSSV probes, although the intensity of the hybridization signals varied. The broad consistency of these data suggests that these WSSV clinical samples from different geographical locations are closely related.

In the subsequent analysis of South Carolina and Texas specimens, 4 of 10 Texas specimens failed to give a positive hybridization signal with the pms146 and pms321 probes, while none of the Texas samples hybridized detectably with the pmh11 probe (Table 3). Examples of the dot hybridization results are shown in Fig. 1.

Analysis of the DNA of WSSV geographical clinical samples by PCR with specific primer sets and restriction fragment length polymorphism

As predicted, the sizes of the 10 amplicons were very close, ranging from 0.9 to 1.5 kbp (Fig. 2), but their restriction profiles varied (Fig. 3). We used these primer pairs to amplify DNA fragments from 71 DNA samples from 7 geographical clinical samples as indicated in Tables 2 & 3. Using the 10 primer pairs and PCR, 10 specific DNA fragments were yielded from the 47 tested DNA samples of 6 WSSV geographical clinical samples (China96-116A, India95-314, crayfish97-25, Thailand95-46, South Carolina97-64, grocery store 96-115). The sizes of major PCR products from 6 WSSV geographical clinical samples using the same primer pair were very similar and showed the expected sizes (Fig. 4). Unlike the other 6 geographical clinical samples, however, only 1 of the 16 DNA samples prepared from the 11 shrimp specimens of the 2 Texas clinical samples yielded PCR products with all 10 primer sets (Texas95-242-J60; Fig. 4, Lane 56). The *Cfo* I, *Hae* III, *Hpa* II, *Rsa* I RFLP profiles of the PCR

Table 2. Analysis of WSSV geographical strains by dot blot hybridization with 11 WSSV probes derived from Taiwan strain. Numbers of plus symbols (i.e. between 1 and 4) corresponds to increasing levels of hybridization intensity on an arbitrary scale. (–) no hybridization. DNA sample names show collection area/individual shrimp no./tissue source for DNA extraction (G: gill; HP: hepatopancreas; Pl: pleopod; MG: midgut; M: muscle; H: hemolymph; Pe: pereiopod); HP/BP: homogenate of hepatopancreas of shrimp infected with *Baculovirus penaei*. Positive control: WSSV Taiwan clinical sample. Negative control: DNA from WSSV-free *Penaeus monodon*. For probes pms: *Penaeus monodon* WSSV *Sal* I fragment, and pmh: *P. monodon* WSSV *Hind* III fragment

DNA samples number and name	Probes											PCR	
	pms473	pms484	pms100	pms321	pms146	pmh2	pmh10	pmh11	pmh13	pmh32	pmh34	Shrimp DNA[b]	WSSV DNA[c]
1. China96-116A/#1/G	++	++	+	+	+	++	+++	+	++	+++	++	+++	+++
2. China96-116A/#1/HP	+++	+	+	+	+	+	+++	+	++	++	+	++	+
3. China96-116A/#1/HP	+++	+	+	+	+	+	+++	+	++	++	+	+++	+++
4. China96-116A/#1/HP	+++	+	+	+	+	+	+++	+	++	++	+	+++	+++
5. China96-116A/#1/Pl	++	+	+	+	+	++	+++	+	++	++	+	+++	+++
6. China96-116A/#2/MG	+++	+	+	+	+	++	+++	+	++	++	+	+++	+++
7. China96-116A/#2/G	++	+	+	+	+	++	+++	+	++	++	+	+++	+++
8. China96-116A/#3/MG	++++	+++	++	++	++	+++	++++	+	+++	++++	+++	+++	+++
9. China96-116A/#3/Pl	++++	+++	++	++	++	+++	++++	+++	+++	++++	+++	+++	+++
10. India95-314/#1/G	+++	+++	++	++	++	+++	++++	+++	++	+++	+++	+++	+++
11. India95-314/#1/HP	+++	+++	++	++	++	+++	++++	+++	++	+++	+++	+++	+++
12. India95-314/#1/Pl	+++	+++	++	++	++	+++	++++	+++	++	+++	+++	+++	+++
13. India95-314/ #2/G	++	+++	++	+++	++	+++	++++	+++	++	+++	+++	+++	+++
14. India95-314/#2/HP	++	+++	++	++	++	+++	++++	+++	++	+++	+++	+++	+++
15. India95-314/#2/Pl	+++	+++	++	+++	++	+++	++++	+++	++	+++	+++	+++	+++
16. India95-314/#3/G	–	+	–	–	–	+	–	–	–	+	–	+++	++
17. India95-314/#3/HP	–	–	+	–	–	–	–	–	–	–	–	+++	+
18. India95-314/#3/Pl	–	–	+	–	–	–	+	–	–	–	–	+++	+
19. Grocery store95-204/#1/M	–	–	–	–	–	–	–	–	–	–	–	+++	–
20. Grocery store95-204/#1/M	–	–	–	–	–	–	–	–	–	–	–	+++	–
21. Grocery store95-204/#2/M	–	–	–	–	–	–	–	–	–	–	–	+++	–
22. Grocery store95-204/#2/M	–	–	–	–	–	–	–	–	–	–	–	+++	–
23. Grocery store95-204/#3/M	–	–	–	–	–	–	–	–	–	–	–	+++	–
24. Grocery store95-204/#3/M	–	–	–	–	–	–	–	–	–	–	–	+++	–
25. Crayfish97-25/#1/G	–	+	–	+	–	+	++	+	+	+	+	+++	++
26. Crayfish97-25/#1/Pl	–	+	–	+	–	+	++	+	+	+	+	+++	++
27. Crayfish97-25/#2/G	++	+++	++	++	++	+++	+++	+++	+++	++	++	+++	+++
28. Crayfish97-25/#2/Pl	++	+++	++	++	++	+++	+++	+++	+++	++	+++	+++	+++
29. Crayfish97-25/#3/G	+++	+++	++	++	++	+++	+++	+++	+++	++	+++	+++	+++
30. Crayfish97-25/#3/Pl	++++	+++	++	+++	++	+++	++++	+++	++++	++++	+++	+++	+++
31. Thailand95-46/#1/B1[a]	+	+	–	+	–	+	++	+	+	+	+	+	++
32. Thailand95-46/#1/B2[a]	+	+	+	+	–	+	++	+	+	+	+	++	++
33. Thailand95-46/#1/B3[a]	+	+	–	+	–	+	++	+	+	+	+	++	+++
34. Thailand95-46/#1/B4[a]	+	+	+	+	+	+	++	+	+	+	+	++	+++
35. South Carolina97-64/#1/H	++	++	+	+	++	++	++	++	++	++	++	++	+++
36. South Carolina97-64/#2/G	+++	+++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
37. South Carolina97-64/#2/G	+++	+++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
38. South Carolina97-64/#2/G	+++	+++	++	+++	++	+++	+++	+++	+++	+++	++++	+++	+++
39. South Carolina97-64/#2/Pe	++	+++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
40. South Carolina97-64/#2/Pe	++	+++	+	+	++	++	+++	+++	+++	+++	++	+++	+++
41. South Carolina97-64/#2/Pe	++	+++	+	++	++	+++	+++	+++	+++	+++	+++	+++	+++
42. South Carolina97-64/#2/Pl	++	+++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
43. South Carolina97-64/#2/Pl	+++	+++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
44. South Carolina97-64/#2/Pl	+++	+++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
45. South Carolina97-64/#2/M	++	+++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
46. South Carolina97-64/#2/M	++	+++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
47. South Carolina97-64/#2/M	++	+++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
HP/BP	–	–	–	–	–	–	–	–	–	–	–	+	–
Positive control	+++	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
Negative control	–	–	–	–	–	–	–	–	–	–	–	+++	–

[a]B1–B4: 4 tissue blocks cut from a mixed tissue mass of shrimp collected from Thailand
[b]Shrimp DNA PCR using 143/145 primer set
[c]1-step WSSV diagnostic PCR using pms146 primer set indicating the likely presence or absence of WSSV in samples

Table 3. Analysis of WSSV geographical strains by dot blot hybridization with 11 WSSV probes derived from Taiwan strain. Number of plus symbols (i.e. between 1 and 4) corresponds to increasing level of hybridization intensity on an arbitrary scale. N.D.: test not done. (–) no hybridization. DNA sample names show collection area/individual shrimp no./tissue sources for DNA extraction (G: gill; Pl: pleopod, M: muscle). Positive control: WSSV Taiwan clinical sample. Negative control: DNA from WSSV-free *Penaeus monodon*

DNA samples number and name	Probes											PCR	
	pms473	pms484	pms100	pms321	pms146	pmh2	pmh10	pmh11	pmh13	pmh32	pmh34	Shrimp DNA[a]	WSSV DNA[b]
48. South Carolina97-64/#3/G	N.D.	N.D.	N.D.	++	++	N.D.	N.D.	++	N.D.	N.D.	N.D.	+	+
49. South Carolina97-64/#3/Pl	N.D.	N.D.	N.D.	++	++	N.D.	N.D.	++	N.D.	N.D.	N.D.	+	+
50. South Carolina97-64/#4/G	N.D.	N.D.	N.D.	++	++	N.D.	N.D.	++	N.D.	N.D.	N.D.	+	+
51. South Carolina97-64/#4/Pl	N.D.	N.D.	N.D.	++	++	N.D.	N.D.	++	N.D.	N.D.	N.D.	+	+
52. Grocery store96-115/#4/Pl	N.D.	N.D.	N.D.	++	++	N.D.	N.D.	++	N.D.	N.D.	N.D.	+	+
53. Grocery store96-115/#4/M	N.D.	N.D.	N.D.	++	++	N.D.	N.D.	++	N.D.	N.D.	N.D.	+	+
54. Grocery store96-115/#5/Pl	N.D.	N.D.	N.D.	++	++	N.D.	N.D.	++	N.D.	N.D.	N.D.	+	+
55. Grocery store96-115/#5/M	N.D.	N.D.	N.D.	++	++	N.D.	N.D.	++	N.D.	N.D.	N.D.	+	+
56. Texas95-242-J60/#1/M	N.D.	N.D.	N.D.	–	–	N.D.	N.D.	–	N.D.	N.D.	N.D.	+	+
57. Texas95-242-J61/#2/M	N.D.	N.D.	N.D.	–	–	N.D.	N.D.	–	N.D.	N.D.	N.D.	+	–
58. Texas95-242-J43/#3/M	N.D.	N.D.	N.D.	–	–	N.D.	N.D.	–	N.D.	N.D.	N.D.	+	–
59. Texas95-242-J43/#4/M	N.D.	N.D.	N.D.	–	–	N.D.	N.D.	–	N.D.	N.D.	N.D.	+	–
60. Texas95-242-J43/#5/M	N.D.	N.D.	N.D.	+	+	N.D.	N.D.	–	N.D.	N.D.	N.D.	+	–
61. Texas95-242-J43/#6/M	N.D.	N.D.	N.D.	+	+	N.D.	N.D.	–	N.D.	N.D.	N.D.	+	–
62. Texas96-7/#7/G	N.D.	N.D.	N.D.	+	+	N.D.	N.D.	–	N.D.	N.D.	N.D.	+	–
63. Texas96-7/#7/Pl	N.D.	N.D.	N.D.	+	+	N.D.	N.D.	–	N.D.	N.D.	N.D.	+	–
64. Texas96-7/#8/G	N.D.	N.D.	N.D.	+	+	N.D.	N.D.	–	N.D.	N.D.	N.D.	+	–
65. Texas96-7/#8/Pl	N.D.	N.D.	N.D.	+	+	N.D.	N.D.	–	N.D.	N.D.	N.D.	+	–
66. Texas96-7/#9/G	N.D.	N.D.	N.D.	+	+	N.D.	N.D.	–	N.D.	N.D.	N.D.	+	–
67. Texas96-7/#9/Pl	N.D.	N.D.	N.D.	+	+	N.D.	N.D.	–	N.D.	N.D.	N.D.	+	–
68. Texas96-7/#10/G	N.D.	N.D.	N.D.	+	+	N.D.	N.D.	–	N.D.	N.D.	N.D.	+	–
69. Texas96-7/#10/Pl	N.D.	N.D.	N.D.	+	+	N.D.	N.D.	–	N.D.	N.D.	N.D.	+	–
70. Texas96-7/#11/G	N.D.	N.D.	N.D.	+	+	N.D.	N.D.	–	N.D.	N.D.	N.D.	+	–
71. Texas96-7/#11/Pl	N.D.	N.D.	N.D.	+	+	N.D.	N.D.	–	N.D.	N.D.	N.D.	+	–
Positive control				++	++			+++				+	–
Negative control				–	–			–				+	–

[a]Shrimp DNA PCR using 143/145 primer set
[b]1-step WSSV diagnostic PCR using pms146 primer set indicating the likely presence or absence of WSSV in samples

products yielded by this Texas clinical sample suggest that it is in fact very similar if not identical to the other 6 WSSV geographic clinical samples. One of these RFLP profile comparisons is shown in Fig. 5. The other Texas specimens were 1-step PCR negative with most of the primer sets (Fig. 4, Lanes 57 to 71), although some of them did yield PCR products in the tests with pmh11F1/R1, pmh13F1/R1, pms54F1/R1, pms94F1/R1, pms98F1/R1, pms120F1/R1, and pms321 F2/R2. Interestingly, the PCR products yielded by the Texas95-242 specimens were always of the anticipated size (Fig. 4, Lanes 56 to 61), while the faint PCR products yielded by the Texas96-7 specimens in the tests with primer sets pmh11FR1/R1, pms94F1/R1, pms98F1/R1 and pms321F2/R2 were all of unexpected sizes (Fig. 4, Lanes 62 to 71). When the internal primer set 98F2/R2 was used to do 2-step PCR, however, all the Texas samples (Lanes 56 to 71) yielded bands of the expected size that were similar to the other 6 geographic clinical samples (Fig. 4). However, the intensity of 2-step PCR product bands yielded by Texas samples (Lanes 57 to 71) was much weaker than 1 Texas sample (Lane 56) and the other 6 geographic clinical samples (Fig. 4), this implies that the amount of virus in Texas samples (57 to 71) was much less than in the other clinical samples. Southern hybridization analysis also revealed that bands of expected size were present in 1-step PCR products of most of the Texas clinical samples (Fig. 6), thereby indicating the existence of WSSV in these specimens. Even so, there were some anomalies: some of the major PCR products of the Texas96-7 specimens (Fig. 6, Lanes 62 to 71) were visible in the Apms98F1/R1 agarose gel but had a smaller than expected size and failed to hybridize with the Apms98F2/R2 probe, while those bands that successfully hybridized with the Apms98F2/R2 probe had the anticipated size, but were invisible in the Apms98F1/R1 agarose gel.

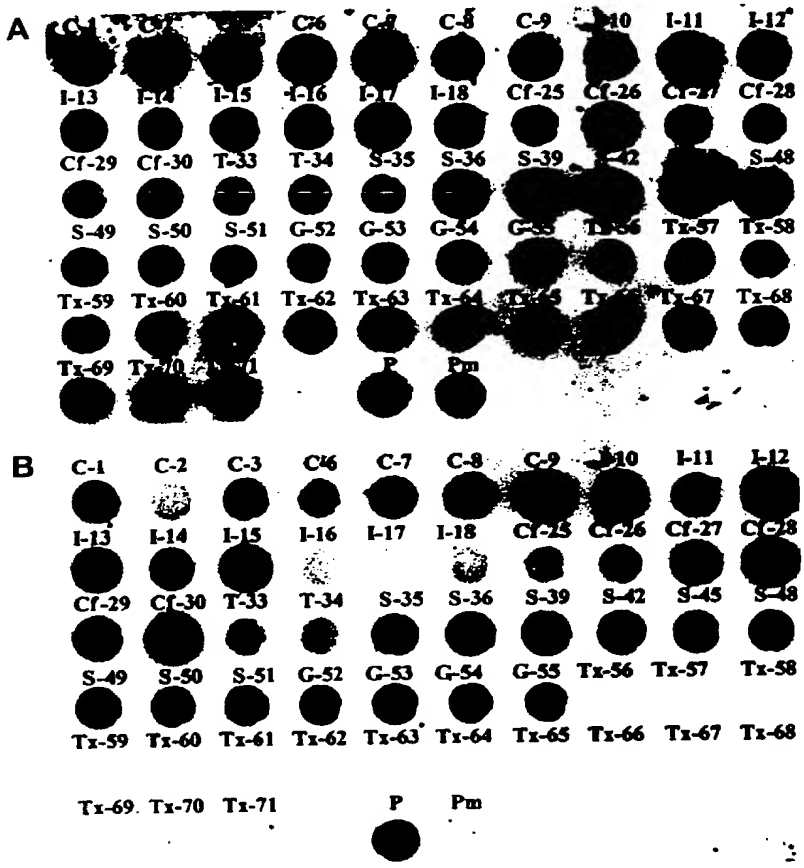

Fig.1. Dot hybridization of WSSV geographical clinical samples with (A) shrimp 18S rRNA gene probe and (B) WSSV probe derived from the amplicon Apms146F1/R1. The geographic origin of each DNA sample is indicated above each dot (C: China; I: India; Cf: Crayfish; T: Thailand; S: South Carolina; G: grocery store; Tx: Texas), and the DNA sample numbers correspond to those used in Tables 2 & 3. P: Taiwan clinical sample. Pm: healthy *Penaeus monodon*

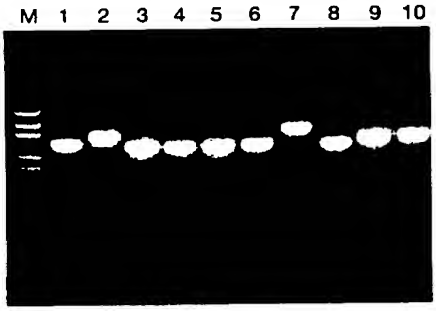

Fig. 2. Ten amplicons, Apmh11F1/R1 (Lane 1), Apmh13F1/R1 (Lane 2), Apms54F1/R1 (Lane 3), Apms94F1/R1 (Lane 4), Apms98F1/R1 (Lane 5), Apms120F1/R1 (Lane 6), Apms146 F1/R1 (Lane 7), Apms321F1/R1 (Lane 8), Apms321F2/R2 (Lane 9), and Apms473F3/R3 (Lane 10), amplified from WSSV Taiwan clinical sample. Lane M: pGEN DNA size marker

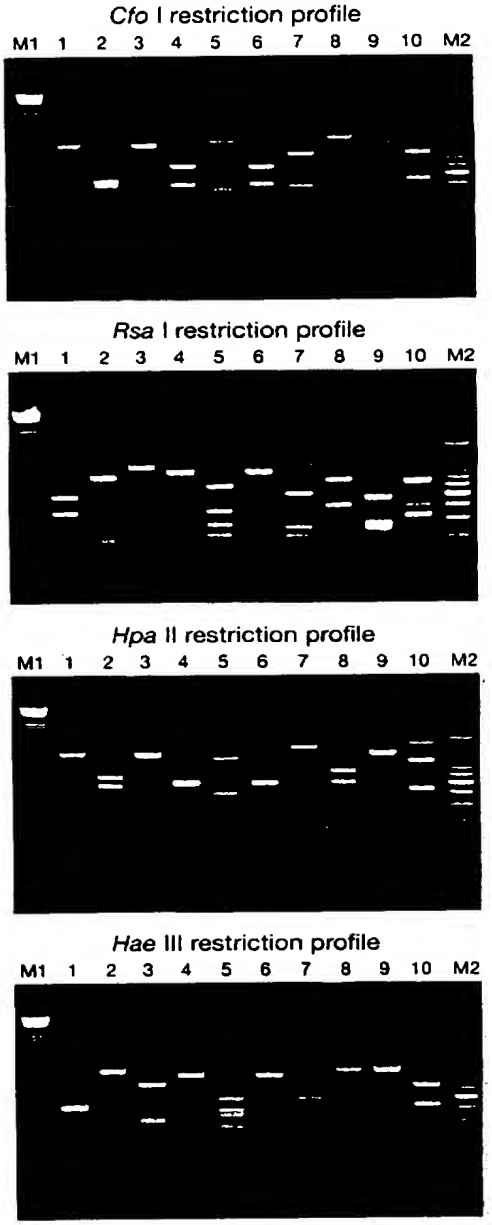

Fig. 3. Restriction profiles of 10 amplicons amplified from WSSV Taiwan clinical sample and cleared with *cfo* I, *Rsa* I, *Hpa* II, and *Hae* III nucleases. (Lane 1) Apms54F1/R1; (Lane 2) Apms94F1/R1; (Lane 3) Apms98F1/R1; (Lane 4) Apms120 F1/R1; (Lane 5) Apms146F1/R1; (Lane 6) Apms321F1/R1; (Lane 7) Apms321F2/R2; (Lane 8) Apms473F3/R3; (Lane 9) Apmh11F1/R1; (Lane 10) Apmh13F1/R1. M1: lambda phage DNA *Hind* III fragments; M2: the 100 bp DNA ladder

When cleaved with *Cfo* I, *Hae* III, *Hpa* II and *Rsa* I restriction endonucleases, the RFLPs of China96-116A, India95-314, crayfish97-25, Thailand95-46, South Carolina97-64, grocery store96-115 and Texas95-242 were very similar, suggesting again that all 7 of these

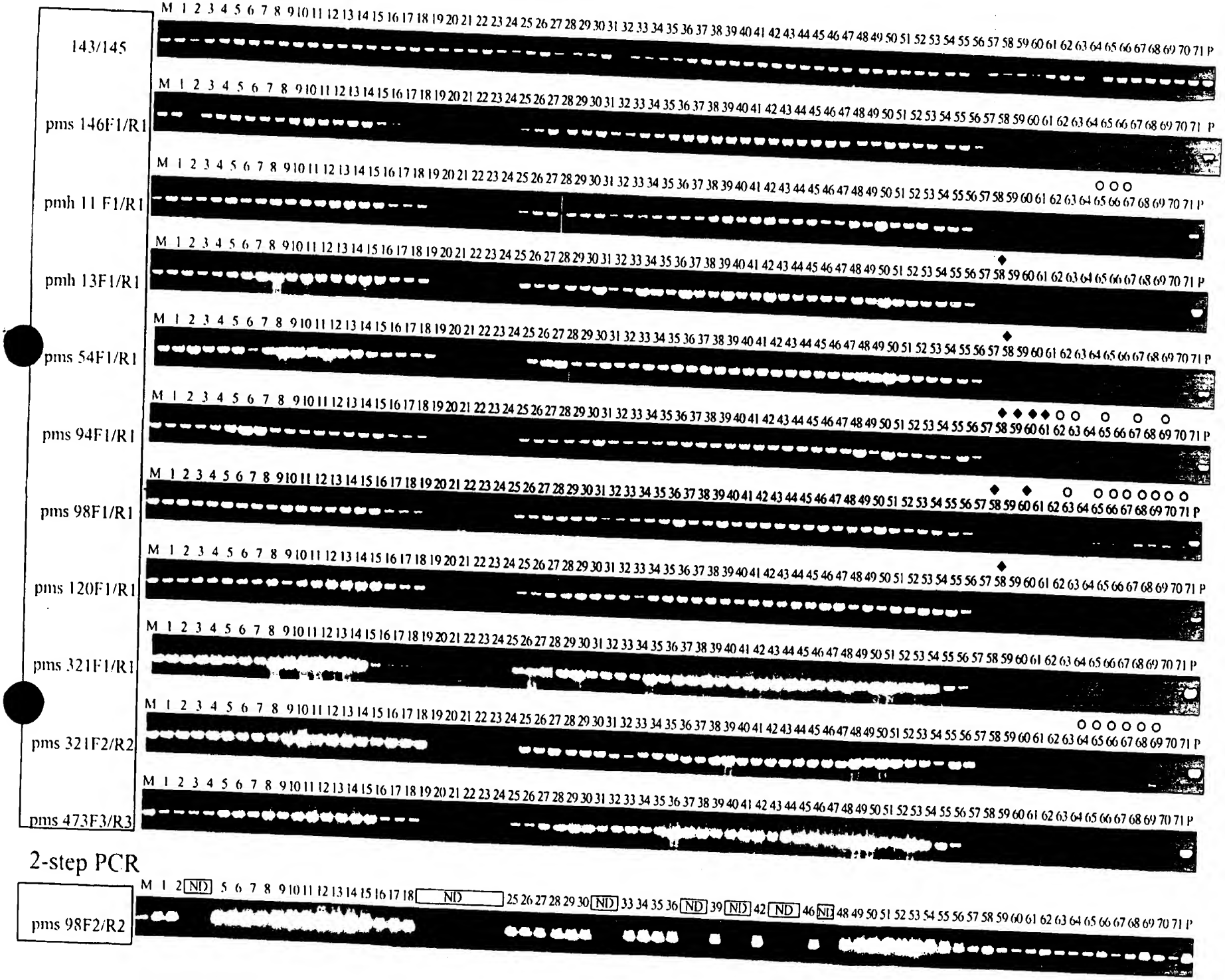

Fig. 4. Amplification of amplicons 143/145, Apms146F1/R1, Apmh11F1/R1, Apmh13F1/R1, Apms54F1/R1, Apms94F1/R1, Apms98 F1/R1, Apms120F1/R1, Apms321F1/R1, Apms321F2/R2, and Apms473F3/R3 by 1-step PCR and Apms98F2/R2 by 2-step PCR from the WSSV geographical clinical samples indicated in Tables 2 & 3. (Lane number corresponds to DNA sample number.) M: PCR size marker. P: Taiwan WSSV. (◆) Faint band with the expected size; (O) band with unexpected size. ND: not detected

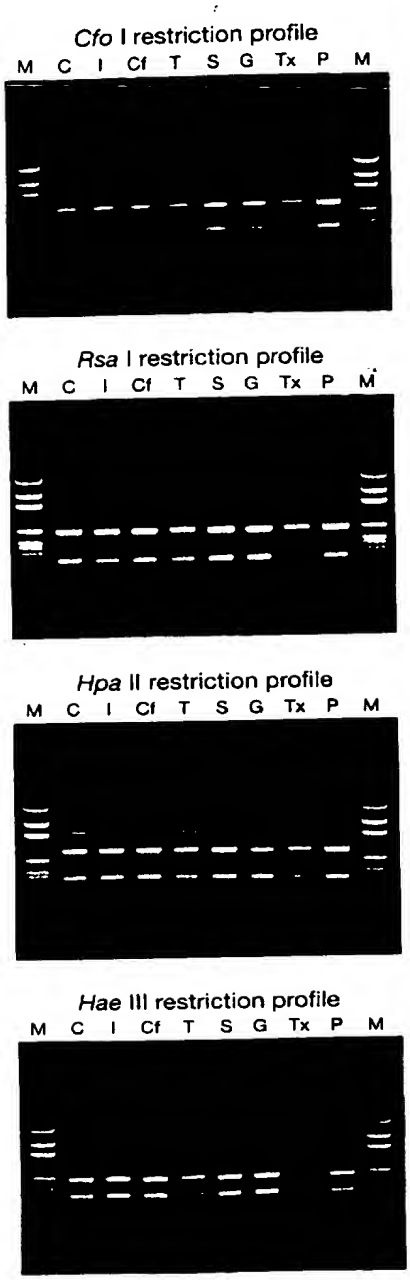

Fig. 5. Restriction profiles of Apmh13F1/R1 amplified from China96-116A (Lane C), India95-314 (Lane I), Crayfish97-25 (Lane Cf), Thailand95-46 (Lane T), South Carolina97-64 (Lane S), grocery store96-115 (Lane G); Texas95-242-J60 (Lane Tx) and Taiwan WSSV (Lane P) cleaved with *Cfo* I, *Rsa* I, *Hpa* II and *Hae* III endonucleases. M: pGEM DNA marker

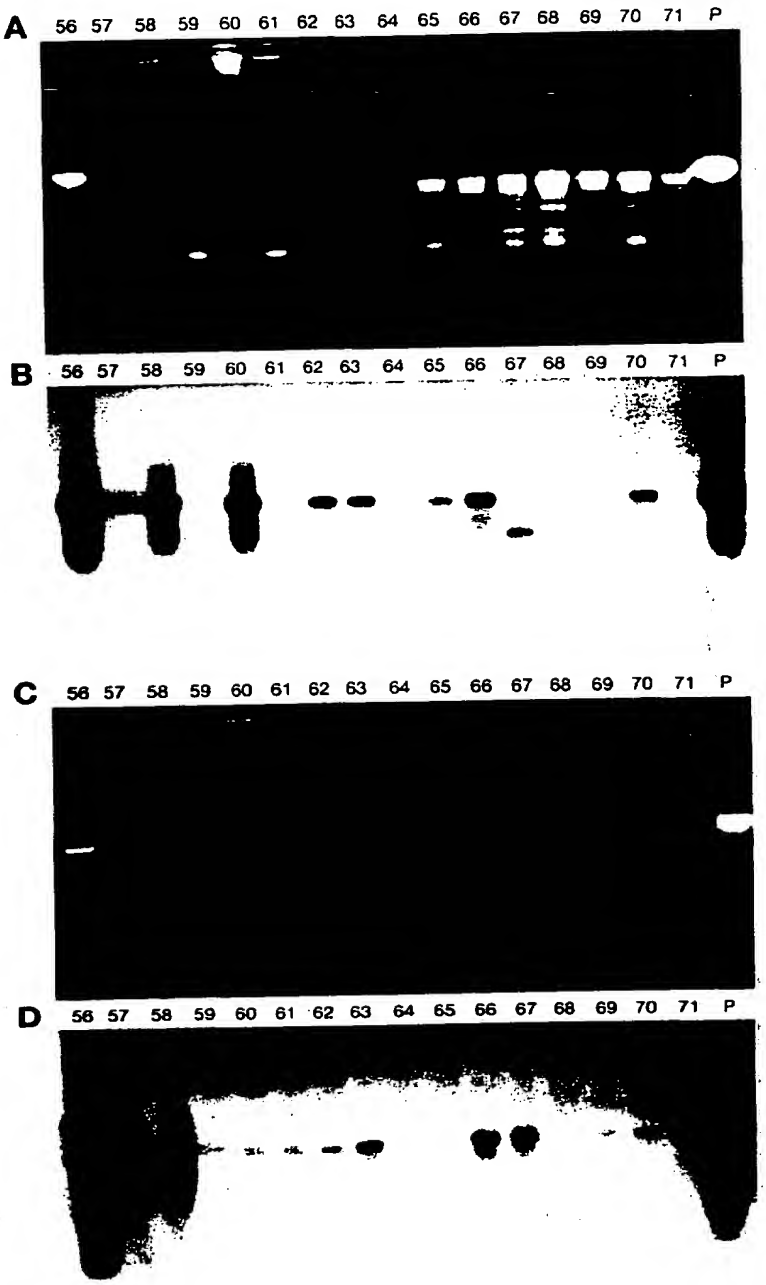

Fig. 6. Southern blot hybridization analysis of PCR products. Ethidium bromide-stained agarose gel of PCR products of Apms98F1/R1 (A) and Apms146F1/R1 (C) are Southern blot hybridized with Apms98 F2/R2 probe (B) and Apms146F2/R2 probe (D), respectively. Lane numbers correspond to the DNA sample numbers used in Table 3

WSSV geographical clinical samples belong to the same virus (group). Examples of RFLP analysis are shown in Fig. 5.

DISCUSSION

As in previous papers, the causal agent of the disease or syndrome is referred to here as WSSV (white spot syndrome virus). However, as several viruses/baculoviruses have been described from the host species investigated here, *Penaeus monodon* (Lightner 1996), it should be borne in mind that the possibility of simultaneous infection by one or more viruses can not be completely excluded in the present study. Accordingly, since we did not purify the virus from the diseased shrimp, the analyzed specimens were therefore termed 'clinical samples' rather than 'virus isolates'. In order that the impurity of the specimens might not lead to difficulties in interpreting the results, the PCR primers used here were derived from the genomic DNA extracted from purified virions of a WSSV Taiwan isolate. These primer sequences (Table 1), and even the fragment sequences from which they were derived, are unique. (unpubl. data from Dr Guang-Hsiung Kou's laboratory). These highly specific primers thus minimize the possibility of any interference that might result from a mixed viral infection.

In the dot hybridization analysis, India95-314#3 and crayfish97-25#1 (Table 2) failed to hybridize with some probes. These anomalous results might have been caused by the virus content in these specimens being very low. Alternatively, some mutants may have existed in shrimp collected from the same geographical location or even from the same culture farm. The PCR tests using 10 primer sets (Fig. 4, Lanes 16 to 18, 25, and 26), however, suggest that the virus in these specimens is similar to other geographic clinical samples (e.g. China96-116, Thailand95-46, South Carolina97-64), so that a low virus content in the samples is very likely to have been the cause of the failure in the hybridization tests.

As shown in Fig. 4, the PCR tests using the 10 primer sets derived from a WSSV Taiwan isolate provided clear evidence that all the specimens of China96-116A, India95-314, crayfish97-25, Thailand95-46, South Carolina97-64, and grocery store96-115 were infected with WSSV or a WSSV-like virus. Almost all the amplicons of the 10 primer sets from these DNA samples appeared as a single band with the expected sizes. Even though a few primer sets gave rise to PCR products from WSSV negative DNA samples (e.g. grocery store95-204 which were somewhat degraded in storage) as either multiple bands (e.g. pms321F1/R1) or single bands of unexpected size (e.g. pms98F1/R1), the results as a whole not only demonstrate the similarity of these clinical samples but also show that these clinical samples can be easily detected using any of the 10 primer sets used for the present study.

The generally good yields seen in Fig. 4 suggest that all the primer sets annealed successfully with the DNA from 48 (DNA samples 1 to 18 and 25 to 55) of WSSV clinical samples from 6 geographical areas (China 96-116A, India95-314, crayfish97-25, Thailand95-46, South Carolina97-64, grocery store96-115). This further suggests that there is very little sequence variation at least within the primer regions of these 6 WSSV geographical clinical samples. Furthermore, the 10 primer sets used in this study correspond to 10 entirely different DNA fragments of the entire WSSV genome. These fragments, of approximately 1 kbp each, therefore constitute a reasonable sampling of the complete genome, so that, since the sizes of the major PCR products from all 6 WSSV geographical clinical samples using the same primer pair were very similar (Fig. 4), it can be argued that substantial similarity exists in the genome structure as a whole.

For 2 Texas clinical samples, however, unexpected reaction patterns were found: although the bands shown by the Texas95-242 specimens were of the expected size, these bands were faint at best and, in many of the PCR tests, no PCR product was yielded at all (Fig. 4, Lanes 57 to 61). We therefore speculate that the virus in Texas95-242 specimens may well be different from the Asian WSSV clinical samples. Further sequence analysis will be required to confirm or refute this hypothesis.

Texas96-7 specimens, on the other hand, yielded faint PCR products in the tests with primer sets pmh11F1/R1, pms94F1/R1, pms98F1/R1 and pms321 F2/R2 but all of them had unexpected sizes (Fig. 4, Lanes 62 to 71). With 2-step PCR (Fig. 4) and Southern hybridization (Fig. 6), however, we found at least some bands with expected sizes in all of the Texas specimens. Although this suggests that the Texas specimens were all infected by WSSV or a WSSV-like virus, the evidence of the PCR reaction patterns argues that the major virus population of Texas WSSV might not be identical to the other geographic clinical samples. In particular, the unexpectedly small size of the major Apms98F1/R1 product yielded by the Texas96-7 specimens (Fig. 6, Lanes 62 to 71) and its failure to hybridize with the Apms98F2/R2 probe suggest this amplicon has little sequence homology to the expected Apms98 F1/R1 PCR product. Before further study can resolve this question, however, the virus will need to be purified. PCR analysis could then check whether the PCR products of unexpected size still exist. If these anomalous amplicons, were still found, we would expect DNA sequencing to reveal a low homology with the

Asian isolates and that the genomic restriction profiles would be distinct. This work would be time consuming, but we believe it is important for the anomalies reported here to be properly accounted for.

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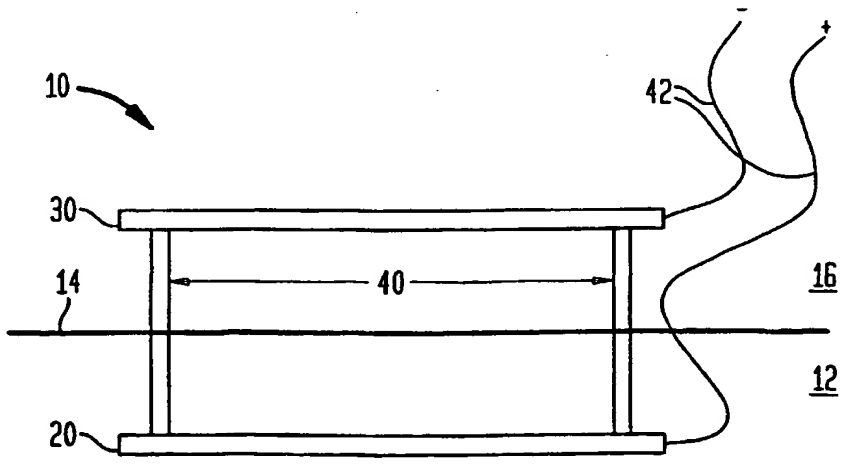
(54) Title: METHOD AND APPARATUS FOR GENERATING POWER FROM VOLTAGE GRADIENTS AT SEDIMENT-WATER INTERFACES



(57) Abstract: A method and apparatus for generating power from voltage gradients at sediment-water interfaces or within stratified euxinic water-columns is provided. Natural voltage gradients typically exist at and about sediment-water interfaces or in isolated water bodies. One electrode (anode) is positioned in the sediment or water just below the redox boundary and the other electrode (cathode) is positioned in the water above the redox boundary over the first electrode. The anode is lower in voltage than the cathode. Current will flow when the electrodes are connected through a load, and near-perpetual generating of worthwhile power may be sustained by the net oxidation of organic matter catalyzed by microorganisms.

WO 01/39306 A1

METHOD AND APPARATUS FOR GENERATING POWER FROM VOLTAGE GRADIENTS AT SEDIMENT-WATER INTERFACES

SPECIFICATION

BACKGROUND OF THE INVENTION

RELATED APPLICATIONS

This application claims the benefit of provisional application U.S. Serial No. 60/166,995 filed November 23, 1999. The disclosure of this application is incorporated herein by reference.

GOVERNMENT RIGHTS

The present invention has been made under the contract with DARPA/ONR, Contract No. N00014-98-1-0690 and the government may have certain rights to the subject invention.

FIELD OF THE INVENTION

The present invention generally relates to generating power from voltage gradients that exist naturally and in association with chemical changes across the interface of water saturated sediments and within stratified water bodies found in aquatic environments.

RELATED ART

Presently, low power (up to 1 Watt continuous average) consuming marine deployed unattended electronics (such as sonar beacons and sensors) are powered by batteries (including conventional lead-acid, alkaline, lithium and seawater), by solar cells, or by direct links to ground available power by means of power conducting cables.

Each of these conventional power sources suffer deficiencies. Conventional batteries are impractical sources of low power for more than one year because they are heavy, bulky, expensive, limited in duration by self-discharge (1-3 years), and commonly fail under deployment conditions in which they are contained in pressure-compensated or pressure resistant vessels.

Seawater batteries specifically designed for low power consuming autonomous marine deployed instrumentation provide 1-2 Watts continuous power for up to three years, limited by depletion of the anode. Solar cells are impractical sources of long-term power for marine deployed instrumentation due to sensitivity to weather and difficulty in maintaining their integrity on the marine surface. Direct cables are impractical sources of low power for marine deployed instrumentation due to cost and logistics.

While it is known that microbial decomposition of organic matter in marine sediments and in stratified water columns will result in the utilization of a succession of oxidizing agents and that this creates a natural voltage gradient, such natural voltage gradients and associated processes are not known to have ever been used as a power source.

What is needed, and has not heretofore been provided, is a method and apparatus of providing power to aquatic deployed devices for prolonged time periods that takes advantages of the natural voltage gradients found at and below sediment-seawater interfaces or within euxinic water bodies.

OBJECTS AND SUMMARY OF THE INVENTION

It is a primary object of the present invention to generate power from voltage gradients established across water saturated sediments and water found in naturally occurring aquatic environments.

It is an additional object of the present invention to provide a method and apparatus for generating energy from microbial established chemical gradients at marine sediment-seawater interfaces.

It is another object of the present invention to provide a method and apparatus for providing power to aquatic deployed equipment for extended periods of time.

It is even another object of the present invention to provide power to aquatic deployed electronics for years.

It is yet another object of the present invention to provide a power source for marine deployed electronics which is naturally replenished.

It is even a further object of the present invention to provide an apparatus for generating power, which power can be used for marine or land applications.

The present invention relates to a method and apparatus for generating power from voltage gradients at sediment-water interfaces (although it is recognized that this method and apparatus may be readily adapted to euxinic water bodies). A natural voltage gradient exists at and about the sediment-water interface because oxygen reduction is found at and about the sediment-water interface, nitrate, manganese and iron reduction frequently occurs in the top few centimeters of the sediment, and sulfate reduction occurs over the next meter or so within the sediment. Single or stacks of inert co-planar electrodes are used to facilitate energy harvesting. One electrode (anode) is positioned in sediment just below the sediment-seawater interface and the other electrode (cathode) is positioned above the sediment and over to the first electrode. The anode is lower in voltage than the cathode. Current will flow between the electrodes when they are connected through a load.

BRIEF DESCRIPTION OF THE DRAWINGS

Other important objects and features of the invention will be apparent from the following Detailed Description of the Invention taken in connection with the accompanying drawings in which:

FIG. 1 is a schematic of the fuel cell for generating energy at an aquatic sediment-water interface according to the present invention.

FIG. 2 is a graph of voltage, current and power between two platinum mesh electrodes having four square inch surface area configured as shown in **FIG. 1** after connection of the electrode leads to a 5,000 ohm resistor.

DETAILED DESCRIPTION OF THE INVENTION

Microbial decomposition of organic matter in an aquatic sediments utilizes a depth-dependent succession of oxidizing agents in which the highest energy liberating oxidants are depleted first. Thus in typical coastal marine sediments, oxygen reduction is found at and above the sediment-water interface, nitrate, manganese, and iron reduction in the top few centimeters, and sulfate reduction over the next meter or so. The depth-dependency of microbe utilized oxidant gives rise to distinctive chemical gradients in sediment pore waters as each oxidant is successively exhausted (O_2, MnO_2, HNO_3, Fe_2O_3, SO_4^{-2}) and its reaction products (H_2O, Mn^{2+}, Fe^{2+}, S^{-2}) produced. A natural consequence of the progressively reduced anoxic sediment layers is a natural voltage gradient. Thus when inert metal electrodes are inserted step-wise into marine sediment from above, a voltage is observed that decreases to as much as 0.8 V relative to a standard reference with increasing depth.

Referring to **FIG. 1**, a schematic of the fuel cell of the present invention is generally indicated at **10**. The fuel cell includes a first electrode (anode) **20** positioned in marine sediment **12** just below the marine sediment-seawater interface **14**. The second electrode (cathode) **30** is positioned over to the first electrode **20** and is positioned in the seawater **16** just above the marine sediment-seawater interface. The relative positions of the anode **20** and cathode **30** are maintained by rig members **40**. The electrodes could be parallel to each other. Electrical leads **42** connect the electrode to a load, not shown. In another embodiment, the electrodes can be positioned in proximity but not parallel to each other. As such, the first electrode is placed in the sediment, but the second electrode could be placed in the water, above the first electrode, at an angle to the first electrode. The second electrode could even be placed at a right angle to the first electrode. A plurality of second electrodes could be lined up and positioned above and perpendicular to the first electrode to optimize power.

By virtue of its position within the microbe established depth-dependent voltage gradient of the sediment **12**, the anode **20** is lower in voltage than the cathode **30**. When connected through a load (i.e., a resistor) a sustained load dependent current flows between the anode **20** and cathode **30** that can be used to

provide electrical power. The electrodes can be solid, mesh, porous or non-porous. Electrode materials include, but are not limited to, platinum, gold, copper, silver, graphite, carbon fibers, which could be contained within fiberglass netting. The size and shape of the electrodes can be varied. Electrical contact are preferably made to each electrode with marine-rated insulated wire and marine-rated insulated epoxy covering conductive epoxy or solder.

Marine sediment/seawater interfaces have been modeled in the laboratory using aquaria containing harvested marine sediments and seawater. At the Naval Research Laboratory, sulfide-rich sediments obtained from a coastal marsh region near Tuckerton, New Jersey, were used. At Rutgers University iron-rich estuarine sediments from Raritan Bay, NJ were used. Prototype power supplies consisting of two electrodes fixed in orientation about sediment-seawater interfaces by non-conductive, rigs were fabricated and demonstrated in both sediment types.

As can be seen with reference to the graph shown in **FIG. 2**, results from prototype testing show the sustainable voltage is created between the electrodes. **FIG. 2** shows voltage, current and power between two platinum mesh electrodes of four square inch surface area configured as represented in **FIG. 1** with a connection of the electrical leads to a 5000 ohm resistor. This current can be used to provide continuous electrical power on the order of 0.5 μ Watts cm^{-2}. Power increases over time and is maintained by microbial oxidation of organic matter in the sediment which regenerates reduced solutes (fuel) that can be oxidized at the bottom electrode (anode) while also sustaining the voltage gradient. Sustainable power cannot be produced in autoclaved (killed) sediments.

The advantage of this power supply over batteries is the prospect of providing necessary power for prolonged periods of time (decades to indefinitely) without the need to replace the power supply because the power supply presumably consumes marine sediment organic matter, which is abundant and replenished by sedimentation and seawater oxygen, which is also abundant and replenished. The advantage of the power supply over the use of solar cells is its insensitivity to weather and its submarine utility. The advantages of the power supply over the use of direct links to ground available power by means of power conducting cables is cost and logistics. The new feature of the present invention

is utilization of marine abundant fuels and oxidant as they are found in the environment in which the power supply is intended for use. Therefore, neither fuel nor oxidant need be deployed or actively transported.

Microbial activity and associated thermodynamically favored biogeochemical reactions create chemical zonation in natural sediments, saturated soils and stratified water columns. The relative concentrations of the chemical constituents in each depth zone determine an electric potential, and vertical profiles of this "redox potential" often decrease by 0.5 to 0.8 volts. In sediments these gradients are often over centimeter scales when measured from the overlying water into the sediment.

Measurements of the concentrations of different redox species within the vicinity of the fuel cell electrodes placed in estuarine and salt marsh sediments indicate that the dominant anode reaction(s) may be:

$$S^{2-} + 4H_2O = SO_4^{2-} + 8H^+ 8e^- \quad \text{or}$$

$$2Fe_4^{2+} + 3H_2O = Fe_2O_3 + 6H^+ + 2e^-$$

whereas the cathode reaction is:

$$2O_2 + 8H^+ + 8e^- = 4H_2O$$

Specific applications of the fuel cell of the present invention include the sustained powering of marine deployed electronics (e.g., sonar beacons and sensors) either as the sole power supply or as supplement to existing power supplies (e.g., recharging batteries). The present invention has application for low power devices, and additionally for generating large amounts of power if done on a larger scale. Further, the generated power can be used for land-based applications where it may be desirable to utilize large components to generate large amounts of power.

Having thus described the invention in detail, it is to be understood that the foregoing description is not intended to limit the spirit and scope thereof. What is desired to be protected by Letters Patent is set forth in the appended claims.

CLAIMS

What is claimed is:

1. An apparatus for generating energy at the interface of aquatic sediment and seawater comprising:

a first anode electrode embedded within the aquatic sediment;

a second cathode electrode positioned within the seawater and above the aquatic sediment;

rig means for maintaining the relative positions of the anode and cathode electrodes; and

electrical leads extending from the anode and cathode electrodes to a load.

2. The apparatus of claim 1 wherein the anode electrode is positioned below the interface of the aquatic sediment and the seawater.

3. The apparatus of claim 1 wherein the electrodes are planar or stacked.

4. The apparatus of claim 3 wherein the cathode electrode is positioned parallel to the anode electrode.

5. The apparatus of claim 3 wherein the cathode electrode is positioned at an angle with respect to the anode electrode.

6. The apparatus of claim 5 wherein the cathode electrode is positioned at a right angle with respect to the anode electrode.

7. The apparatus of claim 6 wherein a plurality of cathode electrodes are positioned over the anode electrode.

8. The apparatus of claim 2 wherein the anode is lower in voltage than the cathode.

9. The apparatus of claim 2 wherein the electrodes comprise platinum.

10. The apparatus of claim 9 wherein the electrodes comprise platinum-mesh.

11. The apparatus of claim 2 wherein the electrodes comprise gold.

12. The apparatus of claim 11 wherein the electrodes comprise gold-mesh.

13. The apparatus of claim 2 wherein the electrodes comprise copper foil.

14. The apparatus of claim 2 wherein the electrodes comprise silver foil

15. The apparatus of claim 2 wherein the electrodes comprise graphite felt.

16. The apparatus of claim 2 wherein the electrodes comprise carbon fibers contained within fiber glass netting.

17. A method of generating power from voltage gradients at interfaces between water and aquatic sediment comprising:

positioning a first electrode in sediment below the interface;

positioning a second electrode in the water over the first electrode; and

connecting electrical leads between the electrodes and a load to create a current between the electrodes.

18. The method of claim 17 further comprising retaining the second electrode in position with respect to the first electrode by interconnecting rigging therebetween.

19. The method of claim 17 further comprising positioning the second electrode parallel to the first electrode.

20. The method of claim 17 further comprising positioning the second electrode at an angle to the first electrode.

21. The method of claim 20 wherein the second electrode is positioned at a right angle to the first electrode.

22. The method of claim 21 further comprising positioning a plurality of second electrodes over the first electrode.

FIG. 1

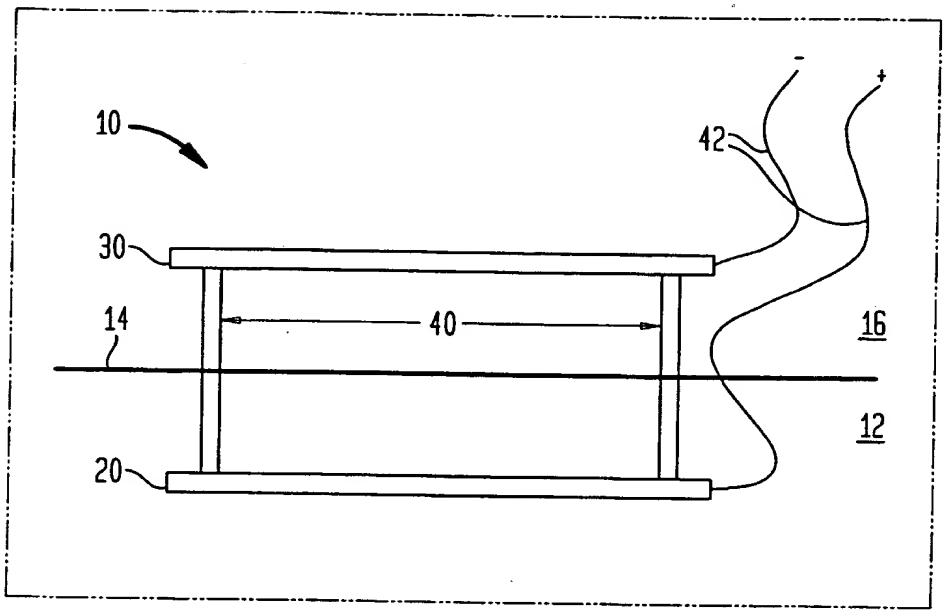

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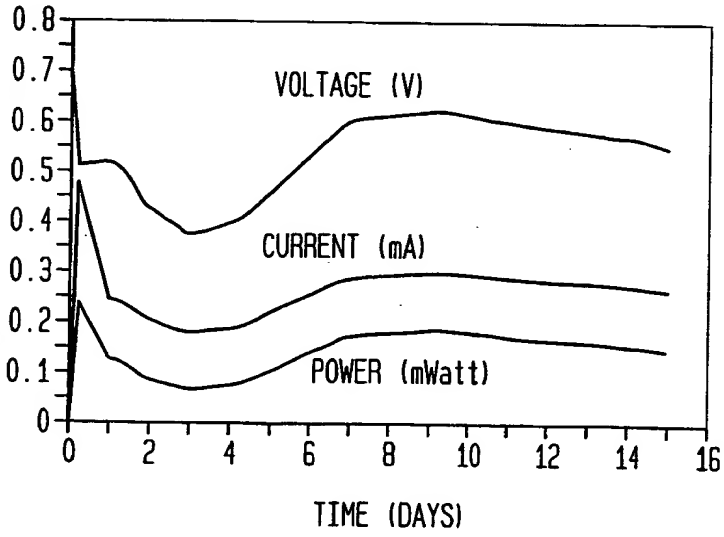
FIG. 2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POWER (mWatt)
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2
4
6
8
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12
14
16
TIME (DAYS)

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER

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US CL :429/119

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 429/119

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST 2.0 (US, JP, EP AND DERWENT)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,770,945 A (CONSTABLE) 23 June 1998.	1
A	US 5,158,838 A (BJORDAL et al.) 27 October 1992.	1
A	US 5,242,768 A (NAGATSUKA et al.) 7 September 1993.	1
A	US 5,427,871 A (GARSHOL et al.) 27 June 1995.	1
A	US 4,278,743 A (THOMPSON) 14 July 1981.	1
A	US 4,085,254 A (ATKINS) 18 April 1978.	1
A	US 5,288,564 A (KLEIN et al.) 22 February 1994.	1

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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